Docket No.: 14677-003US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Klaus GIESE, et al.	)	Confirmation No: 6369
	)	
Application Serial No.: 10/633,630	)	Group Art Unit: 1635
	)	
Filed: August 5, 2003	)	Examiner: Kimberly Chong
	)	
For: INTERFERING RNA MOLECULES	)	

United States Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Declaration under 37 C.F.R. § 1.132

I, Dr. Michael Gait, declare and say:

1. I hold a PhD degree in Chemistry from the University of Birmingham, UK and a BSc in Chemistry, also from the University of Birmingham, UK. A copy of my Curriculum Vitae (CV) and reference list is attached to this declaration as EXHIBIT 1.

2. I am not an employee of or affiliated with Silence Therapeutics. I am being compensated by Proskauer Rose, LLP for the time spent in preparing this Declaration. I have extensive experience in the area of RNA biochemistry, including antisense and RNAi technology. I have published over 200 peer-reviewed publications in the field of oligonucleotide and RNA chemistry, biochemistry and applications in molecular biology. I was working directly in the field of RNAi at the time the captioned application was filed and have further studied degradation of siRNA in serum (see CV and reference list).

3. I have reviewed the specification of the captioned application. In addition to the relevant art, I have, in particular, reviewed the Official Action mailed November 15, 2007, together with the references cited in the Official Action. Specifically, I have reviewed the McSwiggen (US Patent Application US2003/0190635), Tuschl et al. (WO 02/44321), Cook et al. (US Patent 5,955,589) and Damha et al. (US Patent Application US2005/0142535) references.

4. I understand that the currently claimed invention is directed, in part, to a double stranded nucleic acid molecule where each strand contains a stretch of 15-23 ribonucleotides made up of contiguous alternating single unmodified ribonucleotides and single 2'-O-methyl modified ribonucleotides, with the ribonucleotides being linked by natural phosphodiester bonds. The modified ribonucleotides of each strand are "staggered" such that a 2'-O-methyl modified ribonucleotide on one strand base pairs with an unmodified ribonucleotide on the other strand. The double stranded molecule is highly resistant to degradation by nucleases whilst maintaining potent activity in gene silencing. The invention is further directed to the above double stranded nucleic acid molecule having blunt ends.

5. I also understand that the proper standard for considering the state of the RNAi art is from the viewpoint of a person of ordinary skill in the art at the time the invention was made.

6. At the time of the invention, it was known that nucleic acids could be modified by use of various chemical modifications to increase their resistance to cleavage by nucleases. It was also known that such chemical modifications to a nucleic acid could reduce or abolish its functionality (functional activity). It was therefore desirable to chemically modify nucleic acids to increase their resistance to nucleases, but only if the functionality could be maintained.

7. In the case of siRNA molecules, the person of ordinary skill in the art seeking to increase resistance to nucleases whilst retaining siRNA activity is faced with a paradox. This is because the modified double stranded siRNA molecules must be resistant to nucleases present in cells and biological fluids, e.g. serum, without inhibiting the activity of the specific endonuclease in the RISC complex that cleaves the target mRNA as a result of siRNA function. Thus, the effect of chemical modification of an siRNA molecule is highly unpredictable because it

involves decreasing the ability of the nucleic acid to act as a substrate for one or more undesired nucleases present in cells and biological fluids whilst retaining its ability to act as a substrate for the desired endonuclease in the RISC complex. Thus, the property sought to be reduced (nuclease cleavage of a double stranded RNA) is the same property sought to be maintained (nuclease cleavage of a double stranded RNA).

8. Further, the level of unpredictability involved in achieving this desired outcome was even greater, because little or nothing was known about the nuclease or nucleases involved in the unwanted degradation of the double stranded siRNA. Both endonucleases and exonucleases were known to be present in cells and biological fluids at the time of the invention. In 2002, when the captioned application was filed, no nucleases involved in the undesired degradation of double stranded siRNA molecules had been identified, let alone characterized in any manner. Moreover, little was known about RISC, the complex of proteins involved in cleavage of the target mRNA, except that the nuclease in RISC had been partially characterized as Argonaute 2, an endonuclease. A further unknown was how tolerant RISC would be to 2'-O-methyl and other modifications, especially modifications to nucleotides in the region of each strand of siRNA at or near the active cleavage site recognized by the RISC endonuclease. For at least these reasons, the level of unpredictability in identifying modifications to an siRNA molecule that would increase its stability to undesired endonucleases and exonucleases whilst maintaining its functionality to RISC was high. Neither McSwiggen nor Tuschl et al. teach which type or types of undesired nucleases (endonuclease or exonuclease) are responsible for siRNA cleavage in biological fluids (e.g., serum) or in cells.

9. Tuschl et al. (WO 02/44321) states that RNAi activity was abolished if all nucleotides on the sense, antisense or both the sense and antisense strands were substituted with 2'-O-methyl groups (p. 46, lines 13-14 and Figure 14, bars 7-9). Contrary to the examiner's assertions, Tuschl et al. do not describe any 2'-O-methyl modified siRNA that retain activity (p. 46, lines 5-14). Rather, the only modified molecules described by Tuschl et al. that retained RNAi activity contained 2'-deoxy substitutions at the 3' ends (p. 46, lines 8-12 and Figure 14, bars 2-6). Note that the placement of these modifications at the 3' ends is in line with the expectation that undesired nuclease activity on double stranded siRNA might be due to

exonucleases, as was well known for DNA oligonucleotides. Accordingly, the examiner's assertion that there are a "finite number of identified and predictable configurations of 2'-modified and unmodified nucleotides known to impart increased stability and functionality in dsRNA as taught by Tuschl" can apply, at most, to these 2'-deoxy substitutions. Tuschl et al. do not teach any actual or predictable 2'-O-methyl modified configurations that would maintain functionality. In view of Tuschl et al., one of ordinary skill in the art would have concluded that modification of siRNA molecules with 2'-O-methyl groups would reduce or abolish RNAi activity and, accordingly would not have been motivated to make 2'-O-methyl modified siRNA molecules, especially in the region of the active cleavage site recognized by the RISC endonuclease.

10. A group led by Tuschl published an academic paper (Elbashir et al. EMBO J. 20:6877 (2001)) (EXHIBIT 2) corresponding to work disclosed in Tuschl et al. (WO 02/44321). In Elbashir et al., the Tuschl group provides the reader with "The siRNA user guide" (page 6885, column 1). A similar "guide" appears in Tuschl et al. (WO 02/44321) at page 49, line 25, et seq. In both cases, the "guide" begins by pointing out that 2'-deoxy substitutions at the ends of the siRNA duplexes are useful but that "2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNP assembly" (Elbashir et al. at page 6885, last sentence, first full paragraph; Tuschl et al. page 49, line 31 to page 50, line 2). Thus, Tuschl et al. warn against making 2'-O-methyl modifications, predicting that such modifications would reduce functionality. Elbashir et al. further states that the reason for loss of functionality caused by 2'-O-methyl modifications was "probably because methylation of the 2'-hydroxyls blocked hydrogen bond formation or introduced steric hindrance" (page 6886, column 2, last sentence of paragraph bridging columns 1 and 2). In view of the prior art, including Elbashir et al., one of ordinary skill in the art would have expected that making 2'-O-methyl modifications to an siRNA molecule, especially in the region of the active cleavage site recognized by the RISC endonuclease, would either abolish or greatly reduce functionality.

11. That Elbashir et al. remained the state of the art at the time of the captioned application was filed is further supported by Amarzguioui et al. NAR 31(2):589 (2003) (Exhibit 3) who noted that "siRNA with a general 2'-O-methylation in the either strand have no activity"

(page 591, column 1, lines 19-21). Amarzguioui et al. were then motivated to place 2'-O-methyl groups less extensively in the siRNA, but only at the 3' and 5' ends of each strand. Amarzguioui et al. were not motivated to try modifications in the central region and none are described. Similarly, Chiu and Rana, RNA 9:1034 (2003) (Exhibit 4) showed that incorporation of 2'-O-methyl nucleotides fully in either strand "greatly diminished EGFP gene silencing" (page 1037, column 2, line 4) and noted that "the bulkiness of the methyl group would likely be the cause of decreased RNAi activity" (page 1037, column 2, line 11-12). These authors noted that 2'-fluoro pyrimidine residues in both strands generally allowed reasonable functionality of the siRNA by contrast..

12. McSwiggen (US Patent Application US2003/0190635) provides no guidance to one of ordinary skill in the art as to the specific placement of 2'-O-methyl modifications that would both increase stability and maintain functionality. McSwiggen particularly lacks any specific statements or teachings regarding molecules containing 2'-O-methyl modifications within the antisense strand, which is the strand that ultimately binds to the target mRNA and directs its cleavage by the RISC endonuclease. This lack of a teaching or suggestion of making 2'-O-methyl modifications within the antisense strand is best understood when placed in the context of the generally accepted belief at the time of the invention that the structural requirements for the recognition of the antisense strand by RISC were more stringent than for the sense strand. This belief is reflected, for instance, in the comments in Elbashir et al. and Tuschl et al., described above, regarding the effects of 2'-O-methyl modification on protein association for siRNP assembly and in the comment by Elbashir et al. that "the chiral environment is distinct for sense and antisense siRNA, hence their function" (page 6885, column 2, first full paragraph). McSwiggen specifically points out the unpredictability in the state of the art at the time of his filing of siRNA chemical modification using 2'-O-methyl groups with the statement that the art fails to "show to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA" (paragraph [0006]). McSwiggen also fails to provide any data regarding the activity of siRNA molecules that contain 2'-O-methyl modifications within the antisense strand. Even in the sense strand McSwiggen only teaches that pyrimidine nucleotides can be substituted by 2'-O-methyl, not purine.

13. Tuschl et al. at page 3, lines 22-27, states that "if both strands are *exactly* 21 or 22 nt, it is possible to observe *some* RNA interference when both ends are blunt (0 nt overhang)" (emphasis added). One of ordinary skill in the art would have interpreted this statement, when read in the context of the entire document, to mean that blunt end siRNA molecules 21 or 22 nt in length have significantly reduced activity and blunt ended siRNA molecules that are less than 21 nt or greater than 22 nt are not active. This is supported by Elbashir et al., for example at Figure 1, panels F-J, which show that all blunt end molecules have substantially reduced or no activity. Further support is provided on page 6884 which contains the opening statement under the heading "Sequence effects and 2'-deoxy substitutions in the 3' overhang" that reads "The 2 nt 3' overhang is critical for siRNA function" (emphasis added). The Tuschl group reinforces this assertion in the first sentence of the "The siRNA user guide" on page 6885 of Elbashir et al. stating that efficiently silencing siRNA "must be selected to form a 19 bp double helix with 2 nt 3' overhanging ends" (emphasis added).

14. At the time the captioned application was filed it was generally believed that double stranded siRNA was most vulnerable to cleavage by exonucleases (nucleases that degrade from the ends of the nucleic acid), rather than endonucleases (nucleases that cleave within a nucleic acid). Modifications at the ends of a nucleic acid were believed to be sufficient to protect against degradation by exonucleases. Thus, one of ordinary skill would not have been motivated to make modifications, particularly 2'-O-methyl modifications, to an siRNA molecule, other than possibly to the ends, because it was believed that modifications at positions other than at the ends of the molecules would severely reduce or abolish functionality. That exonucleases were thought primarily responsible for siRNA degradation at the time of the invention is shown by Soutschek et al. Nature 432(11):173-178 (2004) (EXHIBIT 5) which states "exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum."

15. It also should be noted that the effects of other types of potential 2'-modification, such as 2'-fluoro modification, are not predictive of the results of 2'-O-methyl modifications because the various modifying groups differ in their chemical and physical properties. For example, a 2'-fluoro group is much smaller than a 2'-O-methyl group and would thus not be expected to have the same issues with steric hindrance, as supported by Chiu and Rana.

16. Cook et al. (US Patent 5,955,589) and Damha et al. (US Patent Application US2005/0142535) provide no evidence against the position that at the time the captioned application was filed, one of ordinary skill in the art would have expected that an siRNA molecule containing 2'-O-methyl modifications within the central portion of the antisense strand involved in RISC binding and target cleavage, would have either greatly reduced or abolished functionality of the siRNA molecule.

17. Cook et al. do not report a double stranded RNA molecule, but only on a single stranded DNA molecule and how it may be substituted by 2'-O-methyl residues. It was known at the time the captioned application was filed that functionality of an antisense DNA oligonucleotide was reduced or abolished if the middle portion of the antisense DNA oligonucleotide, i.e., the portion recognized and cleaved by RNase H, was modified with 2'-O-methyl groups. Functionality was not affected if the same modifications were made at the ends of the antisense oligonucleotide. This gave rise to the creation of gapmers, such as those taught by Cook et al. With gapmers, the end portions, but not the middle portion, can be modified with 2'-O-methyl groups. Cook et al. even demonstrates how 2'-O-methyl modifications across the middle portion of an antisense oligonucleotide greatly reduce antisense functionality in Figure 2 (oligos 3975 and 3979). Cook et al. therefore teach that the central region of the DNA that is recognized by RNase H, an enzyme that recognizes a DNA/RNA hybrid (RNA target), cannot be substituted by 2'-O-methyl.

18. Damha et al. also do not report on a double stranded RNA molecule but instead show a single stranded DNA molecule and how it may be substituted by 2'-O-methyl. It was further known that antisense DNA oligonucleotides with alternating 2'-O-methyl modifications that spanned the middle portion of the antisense oligonucleotide also significantly reduced or abolished functionality. For example, Damha et al. states that an antisense oligo with alternating 2'-O-methyl modifications spanning the entire oligonucleotide "showed only poor ability to elicit RNase H degradation of target RNA (Fig. 3)." (paragraph [0149]) This severe reduction in functionality is demonstrated in Figure 3, sequence 8 with the sequence shown in Table 1 (p. 10, bottom of column 1).

19. Thus, together, Cook et al. and Damha et al. show that alternating 2'-O-methyl modifications of a nucleic acid have the same or similar effect on abolishing or severely reducing functionality as does 2'-O-methyl modifications on all nucleotides. Both fully modified and alternating modified antisense oligonucleotides similarly fail to elicit cleavage of a target mRNA by an endonuclease. Elbashir et al. shows that 2'-O-methyl modifications on all nucleotides of either siRNA strand fail to elicit cleavage by the RISC endonuclease of a target mRNA. One of ordinary skill in the art would have expected alternating 2'-O-methyl modifications of an siRNA molecule to have the same or similar effect as a fully 2'-O-methyl modified strand of an siRNA molecule, with the result of either severely reducing or abolishing cleavage of a target mRNA.

20. In summary, for the reasons set forth above, the state of the art of chemically modifying siRNA in a manner that would both increase resistance to cleavage by nucleases and maintain functionality at the time of the invention was unpredictable.

21. One of ordinary skill in the art would further not have been motivated to make the internal 2'-O-methyl modifications of the claimed nucleic acid because at the time of the invention it was believed that chemical protection against exonucleases rather than endonucleases was required to obtain a double stranded siRNA with suitable stability for use in biological fluids or cells.

22. In view of state of the art at the time of the invention, one of ordinary skill in the art would not have been motivated to make the nucleic acids claimed in the captioned application because alternating 2'-O-methyl modifications would have been expected to greatly reduce or abolish functionality (in terms of the ability of the siRNA to elicit cleavage of a target mRNA by RISC) in a manner similar to what was shown for full modification across the RISC recognition and cleavage site.

23. One of ordinary skill in the art would still further not have been motivated to make 2'-O-methyl modifications to the antisense strand because at the time of the invention it

was believed that the antisense strand would be far less tolerant to modifications than the sense strand.

24. For these reasons, the ability of the claimed nucleic acid having alternating 2'-O-methyl modifications to be both highly resistant to nuclease activity as well as retaining a high level of RNAi activity, as shown, for example, in Figure 15 is unexpected.

25. In addition, the statements by Elbashir et al. described above that overhangs are "critical" for RNAi activity and that efficiently silencing siRNA "must be selected to form a 19 bp double helix with 2 nt 3' overhanging ends" mean that the ability of the claimed nucleic acid having blunt ends to retain full RNAi activity, as shown, for example, in Figure 16, oligonucleotides 155/156 and 161/162, is unexpected.

26. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

Date: February 4th 2008

Dr. Mike Gait

EXHIBIT 1 of

EXHIBIT B

CURRICULUM VITAE

Michael John Gait

Date and Place of Birth: 23rd December, 1948. Calcutta, India
Citizenship: British

PRESENT EMPLOYMENT

4/94 - MRC Programme Leader
4/87 - 3/94 Senior Staff Scientist
9/75 - 4/87 Staff Scientist (tenured 1980)

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EDUCATION

1973 PhD, Chemistry, University of Birmingham, England
Supervisors: Prof A S Jones, Prof. R T Walker. Subject: Nucleic Acids
1970 BSc, Chemistry (Class I) University of Birmingham, England

OTHER EMPLOYMENT EXPERIENCE

9/82 - 9/83 Research Associate, Collaborative Research Inc., (leave of absence)
Waltham, Massachusetts, USA
9/73 - 8/75 Research Associate, Massachusetts Institute of Technology,
Cambridge, Massachusetts, USA (Department of Chemistry)
Project Director: Prof H. G. Khorana

CONSULTANCIES

04/07 - GlaxoSmithKline
09/06 - Panagene Inc. (Scientific Advisory Board)
7/97 - 6/03 RiboTargets Ltd, Cambridge, UK (Scientific Advisory Board)
7/89 - 6/03 Gilead Sciences, Foster City, CA, USA (Scientific Advisory Board)
5/94 - 4/95 Glaxo Group Research, Greenford, UK
9/86 - 9/92 British Bio-Technology Ltd, UK
9/86 -10/88 Integrated Genetics/Gene-Trak Systems, Framingham, USA
8/81 - 4/82 Transgene, Strasbourg, France
3/81 - 3/82 Celltech Ltd, Slough, UK
10/79 - 4/85 Collaborative Research Inc., Waltham, MA, USA

AWARDS

Winner of the 2003 RSC Award in Nucleic Acids Chemistry
Elected an EMBO member 2006

SCIENTIFIC SOCIETIES

Royal Society of Chemistry: Associate Member 1973-1987
Fellow of the RSC 2002-
Chemical Biology Forum Exec Committee Member 2003-2007
Awards Committee 2007-
Nucleic Acids Group Committee 1982-1994 and 2000-
(Secretary/Treasurer 1988-1992 Chairman 2000-2003)

M J Gait

PUBLICATIONS

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11. A polyamide support for oligonucleotide synthesis. M.J. Gait and R.C. Sheppard, *J. Amer. Chem. Soc.*. (1976) **98**, 8514-8516.

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EXHIBIT 2 of

EXHIBIT B

The EMBO Journal Vol. 20 No. 23 pp. 6877–6888, 2001

Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate

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Duplexes of 21–23 nucleotide (nt) RNAs are the sequence-specific mediators of RNA interference (RNAi) and post-transcriptional gene silencing (PTGS). Synthetic, short interfering RNAs (siRNAs) were examined in *Drosophila melanogaster* embryo lysate for their requirements regarding length, structure, chemical composition and sequence in order to mediate efficient RNAi. Duplexes of 21 nt siRNAs with 2 nt 3′ overhangs were the most efficient triggers of sequence-specific mRNA degradation. Substitution of one or both siRNA strands by 2′-deoxy or 2′-*O*-methyl oligonucleotides abolished RNAi, although multiple 2′-deoxynucleotide substitutions at the 3′ end of siRNAs were tolerated. The target recognition process is highly sequence specific, but not all positions of a siRNA contribute equally to target recognition; mismatches in the centre of the siRNA duplex prevent target RNA cleavage. The position of the cleavage site in the target RNA is defined by the 5′ end of the guide siRNA rather than its 3′ end. These results provide a rational basis for the design of siRNAs in future gene targeting experiments.

Keywords: PTGS/RNA interference/small interfering RNA

Introduction

Post-transcriptional gene silencing (PTGS) mediated by double-stranded (ds) RNA represents an evolutionarily conserved cellular defence mechanism for controlling the expression of alien genes in protists, filamentous fungi, plants and animals (Fire, 1999; Bass, 2000; Cogoni and Macino, 2000; Carthew, 2001; Hammond *et al.*, 2001b; Sharp, 2001; Tuschl, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). It is believed that random integration of alien genes (such as transposons) or viral infection causes production of dsRNA, which activates sequence-specific degradation of homologous single-stranded mRNA or viral genomic RNA, thereby preventing expression or replication of the foreign genetic material. The dsRNA is used as the guide RNA in this sequence-specific RNA degradation process. In some cases, dsRNA may also be involved in amplification of the silencing signal important for systemic spread (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998) or long-term maintenance of silencing (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Smardon *et al.*, 2000). In animals, the dsRNA-triggered silencing effect is referred to as RNA interference (RNAi; Fire *et al.*, 1998).

One important feature of the mechanism of RNAi is the processing of long dsRNAs into duplexes of 21–25 nucleotide (nt) RNAs. These short RNA products were first detected in plant tissues that exhibited transgene- or virus-induced PTGS (Hamilton and Baulcombe, 1999), but were also found later in fly embryos and worms injected with long dsRNAs (Parrish *et al.*, 2000; Yang *et al.*, 2000) or in extracts from *Drosophila melanogaster* Schneider-2 (S2) cells that were transfected with dsRNA (Hammond *et al.*, 2000). The processing reaction of long dsRNAs to 21–23 nt RNAs was first recapitulated *in vitro*, in extracts prepared from *D.melanogaster* embryos (Zamore *et al.*, 2000) and later in extracts from S2 cells (Bernstein *et al.*, 2001). In the embryo lysate, it was observed that the target mRNA was cleaved in ~21 nt intervals (Zamore *et al.*, 2000) and that synthetic 21 and 22 nt RNA duplexes added to the lysate were able to guide efficient sequence-specific mRNA degradation, while duplexes of 30 bp dsRNA were inactive (Elbashir *et al.*, 2001b). The 21 nt RNA products were therefore named small interfering RNAs or silencing RNAs (siRNAs).

A ribonuclease III enzyme, dicer, is required for processing of long dsRNA into siRNA duplexes (Bernstein *et al.*, 2001). It was recently shown that dicer has an additional cellular function and is also required for excision of 21 and 22 nt small temporal RNAs (stRNAs) from ~70 nt stable stem–loop precursors (Grishok *et al.*, 2001; Hutvágner *et al.*, 2001). These tiny expressed RNA molecules are important regulators of developmental timing and control the translation of downstream regulatory genes (Ambros, 2000; Moss, 2000; Pasquinelli *et al.*, 2000). stRNAs are different from siRNAs in that the target mRNA is not degraded during silencing (Wightman *et al.*, 1993; Olsen and Ambros, 1999) and they are single stranded (Reinhart *et al.*, 2000), while siRNAs are believed to be double stranded (Elbashir *et al.*, 2001b; Hutvágner *et al.*, 2001).

In RNAi, a siRNA-containing endonuclease complex cleaves a single-stranded target RNA in the middle of the region complementary to the 21 nt guide siRNA of the siRNA duplex (Elbashir *et al.*, 2001b). This cleavage site is one helical turn displaced from the cleavage site that produced the siRNA from long dsRNA, suggesting dramatic conformational and/or compositional changes after processing of long dsRNA to 21 nt siRNA duplexes. The target RNA cleavage products are rapidly degraded because they either lack the stabilizing cap or poly(A) tail. A protein component of the ~500 kDa endonuclease or

RNA-induced silencing complex (RISC) was recently identified and is a member of the argonaute family of proteins (Hammond *et al.*, 2001a); however, it is currently unclear whether dicer is required for RISC activity.

It is also unknown whether RISC contains single- or double-stranded siRNAs. By analogy to stRNA excision, it may be envisaged that only one of the strands of a siRNA duplex is incorporated into a catalytic siRNP, but because of the symmetry of the siRNA duplex, two approximately equal populations of sense and antisense strand-containing catalytic siRNPs are produced. Synthetic siRNA duplexes cleaved sense as well as antisense target RNAs in the middle of the region covered by the siRNA duplex in *D.melanogaster* lysate (Elbashir *et al.*, 2001b). However, longer dsRNAs did not produce symmetric sense and antisense target RNA cleavage sites in embryo lysate (Elbashir *et al.*, 2001b), suggesting that the direction of processing of long dsRNA defined which of the strands of the resulting siRNA duplex could be used for guiding target degradation. Some protein, involved in the production of the 21 nt siRNA duplexes, may be deposited on the siRNA duplex to mark the strand that is going to be used for guiding target RNA cleavage.

Despite the lack of profound mechanistic understanding, RNAi has rapidly developed into an important tool for reverse genetics and has been widely applied in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gönczy *et al.*, 2000; Piano *et al.*, 2000; Maeda *et al.*, 2001), as well as in insects (see references in Lam and Thummel, 2000) and insect cell lines (Clemens *et al.*, 2000; Hammond *et al.*, 2000; Ui-Tei *et al.*, 2000). RNAi has also been shown to occur in a variety of vertebrates by targeting of mRNAs important for embryonic development. In differentiated mammalian cells, dsRNAs with >30 bp generally activate the interferon response, which leads to a global shut-off in protein synthesis as well as non-specific mRNA degradation (Stark *et al.*, 1998). This unspecific response to long dsRNAs can be bypassed using 21 nt siRNA duplexes, resulting in specific knock-down of the expression of the targeted gene (Elbashir *et al.*, 2001a; Hutvágner *et al.*, 2001), providing a new method for analysis of mammalian gene function in cultured cells.

Here we describe the results of a systematic analysis of the length, secondary structure, sugar backbone and sequence specificity of siRNA duplexes for RNAi, using the established *D.melanogaster* embryo *in vitro* system. The most potent siRNA duplexes are 21 nt long, comprising a 19 nt base-paired sequence with 2 nt 3′-overhanging ends. The 5′ end of the target-complementary siRNA strand (guide siRNA) sets the ruler to define the position of target RNA cleavage. Furthermore, we find that target recognition is extremely specific, as even single nucleotide mismatches between the siRNA duplex and the target mRNA abolish interference. These results provide a rational basis for the design of siRNAs for future gene targeting experiments.

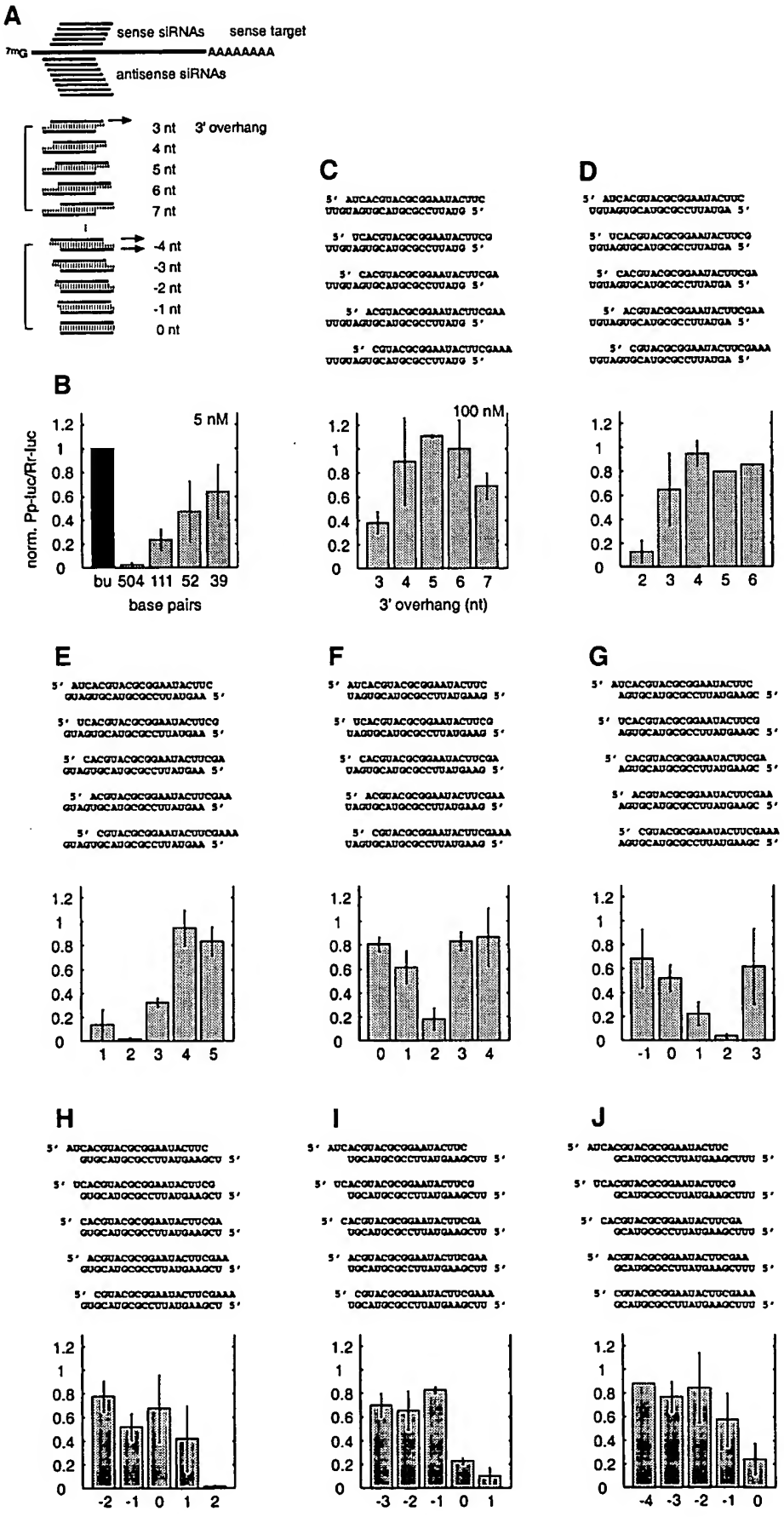
Results

Variation of the 3′ overhang in duplexes of 21 nt siRNAs

We reported previously that two or three unpaired nucleotides at the 3′ end of siRNA duplexes were more efficient in target RNA degradation than blunt-ended duplexes (Elbashir *et al.*, 2001b). To perform a more comprehensive analysis of the function of the terminal nucleotides, we synthesized five 21 nt sense siRNAs, each displaced by one nucleotide relative to the target RNA, and eight 21 nt antisense siRNAs, each displaced by one nucleotide relative to the target (Figure 1A). By combining these sense and antisense siRNAs, a series of eight siRNA duplexes with symmetric overhanging ends were generated spanning a range from 7 nt 3′ overhang to 4 nt 5′ overhang. The interference was measured using the dual luciferase assay system (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). siRNA duplexes were directed against firefly luciferase mRNA and sea pansy luciferase mRNA was used as internal control. The luminescence ratio of target to control luciferase activity was determined in the presence of siRNA duplex and was normalized to that observed in its absence. For comparison, the interference ratios of long dsRNAs (39–504 bp) are shown in Figure 1B (Elbashir *et al.*, 2001b). The interference ratios were determined at concentrations of 5 nM for long dsRNAs (Figure 1A) and at 100 nM for siRNA duplexes (Figure 1C–J). The 100 nM concentration of siRNAs was chosen because complete processing of 5 nM 504 bp dsRNA would result in 120 nM total siRNA duplexes.

The ability of 21 nt siRNA duplexes to mediate RNAi is dependent on the number of overhanging nucleotides or base pairs formed. Duplexes with 4–6 3′-overhanging nucleotides were unable to mediate RNAi (Figure 1C–F), as were duplexes with two or more 5′-overhanging nucleotides (Figure 1G–J). The duplexes with 2 nt 3′ overhangs were most efficient in mediating RNA interference, although the efficiency of silencing was also sequence dependent and up to 12-fold differences were observed for different siRNA duplexes with 2 nt 3′ overhangs (compare Figure 1D–H). Duplexes with blunted ends, 1 nt 5′ overhang or 1–3 nt 3′ overhangs were sometimes functional and sometimes completely inactive. The small silencing effect observed for the siRNA duplex with 7 nt 3′ overhang (Figure 1C) may be due to an antisense effect of the long 3′ overhang rather than to

Fig. 1. Variation of the 3′ overhang of duplexes of 21 nt siRNAs. (**A**) Outline of the experimental strategy. The capped and polyadenylated sense target mRNA is depicted and the relative positions of sense and antisense siRNAs are shown. Eight series of duplexes according to the eight different antisense strands were prepared. The siRNA sequences and the number of overhanging nucleotides were changed in 1 nt steps. (**B**) Normalized relative luminescence of target luciferase (*Photinus pyralis*, Pp-luc) to control luciferase (*Renilla reniformis*, Rr-luc) in *D.melanogaster* embryo lysate in the presence of 5 nM blunt-ended dsRNAs (Elbashir *et al.*, 2001b). The luminescence ratios determined in the presence of dsRNA were normalized to the ratio obtained for a buffer control (bu; black bar). Normalized ratios less than 1 indicate specific interference. (**C–J**) Normalized interference ratios for eight series of 21 nt siRNA duplexes. The sequences of siRNA duplexes are depicted above the bar graphs. Each part shows the interference ratio for a set of duplexes formed with a given antisense guide siRNA and five different sense siRNAs. The number of overhanging nucleotides (3′ overhang, positive numbers; 5′ overhang, negative numbers) is indicated on the *x*-axis. Data points were averaged from at least three independent experiments. Error bars represent standard deviations.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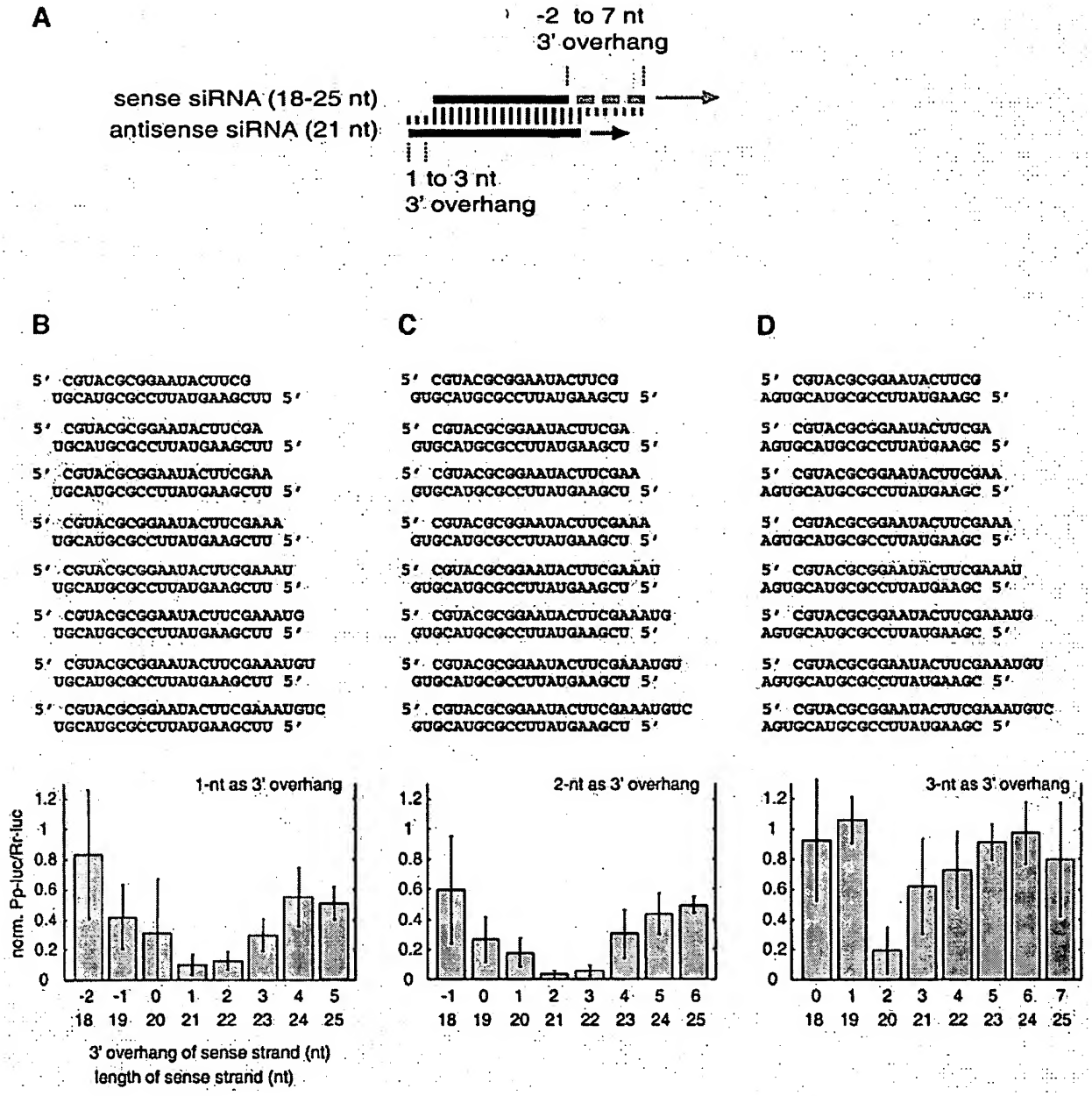


Fig. 2. Variation of the length of the sense strand of siRNA duplexes. (**A**) Representation of the experiment. Three 21 nt antisense strands were paired with eight sense siRNAs. The siRNAs were changed in length at their 3′ end. The 3′ overhang of the antisense siRNA was 1 nt (**B**), 2 nt (**C**) or 3 nt (**D**), while the sense siRNA overhang was varied for each series. The sequences of the siRNA duplexes and the corresponding interference ratios are indicated.

RNAi. Comparison of the efficiency of RNAi between long dsRNAs (Figure 1B) and the most effective 21 nt siRNA duplexes (Figure 1E, G and H) indicates that a single siRNA duplex at 100 nM concentration can be as effective as 5 nM 504 bp dsRNA.

Length variation of the sense siRNA paired to an invariant 21 nt antisense siRNA

In order to investigate the effect of the length of siRNAs on RNAi, we prepared three series of siRNA duplexes, combining three 21 nt antisense strands with eight 18–25 nt sense strands. The 3′ overhang of the antisense siRNA was fixed to 1, 2 or 3 nt in each siRNA duplex series, while the sense siRNA was varied at its 3′ end (Figure 2A). Independently of the length of the sense siRNA, we found that duplexes with 2 nt 3′ overhang of antisense siRNA (Figure 2C) were more active than those with 1 or 3 nt 3′ overhang (Figure 2B and D). In the first series, with 1 nt 3′ overhang of antisense siRNA, duplexes with 21 and 22 nt sense siRNAs, carrying a 1 and 2 nt 3′ overhang of sense siRNA, respectively, were most active. Duplexes with 19–25 nt sense siRNAs were also able to mediate RNA, but to a lesser extent. Similarly, in the second series, with 2 nt overhang of antisense siRNA, the 21 nt siRNA duplex with 2 nt 3′ overhang was most active and any other combination with the 18–25 nt sense siRNAs was active to

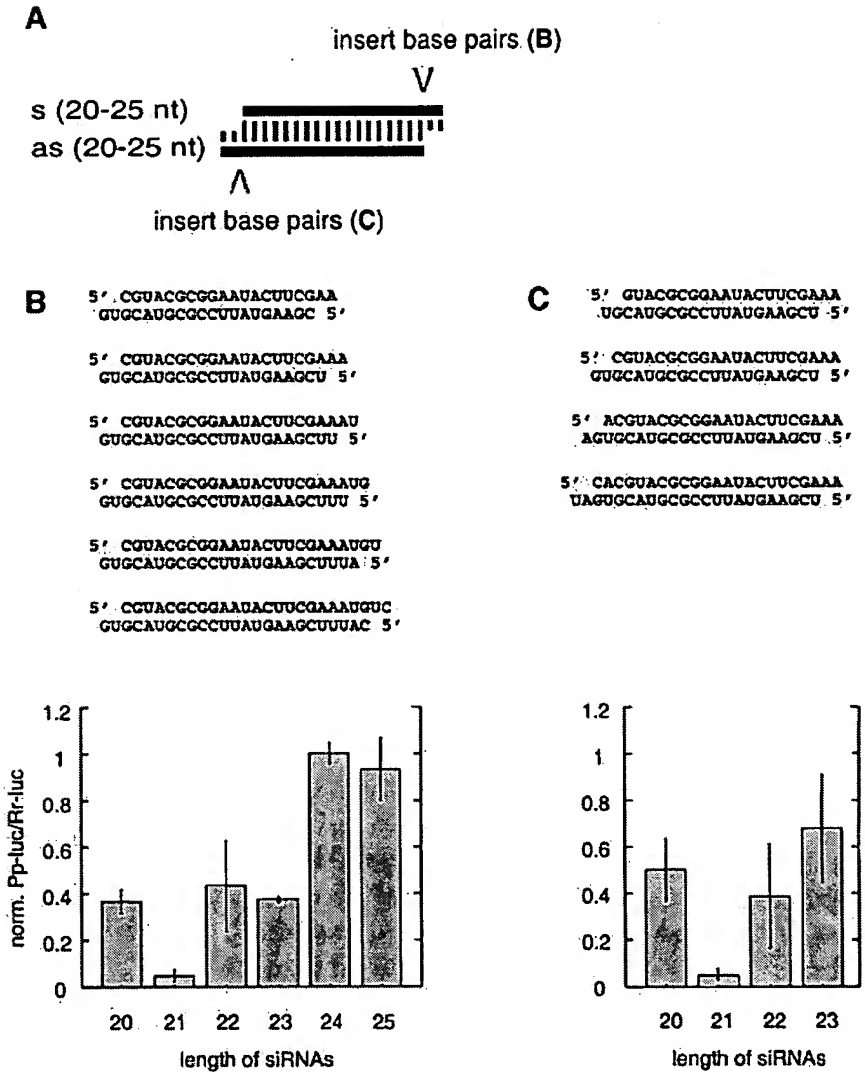


Fig. 3. Variation of the length of siRNA duplexes with preserved 2 nt 3′ overhangs. (A) Graphic representation of the experiment. The 21 nt siRNA duplex is identical in sequence to the one shown in Figures 1H and 2C. The siRNA duplexes were extended to the 3′ side of the sense siRNA (B) or the 5′ side of the sense siRNA (C). The siRNA duplex sequences and the respective interference ratios are indicated.

a significant degree. In the last series, with 3 nt antisense siRNA 3′ overhang, only the duplex with a 20 nt sense siRNA and 2 nt sense 3′ overhang was able to reduce target RNA expression. Together, these results indicate that the length of the siRNA as well as the length of the 3′ overhang are important, and that duplexes of 21 nt siRNAs with 2 nt 3′ overhang are optimal for RNAi.

Length variation of siRNA duplexes with a constant 2 nt 3′ overhang

We then examined the effect of simultaneously changing the length of both siRNA strands by maintaining symmetrical 2 nt 3′ overhangs (Figure 3A). Two series of siRNA duplexes were prepared, including the 21 nt siRNA duplex of Figure 1H as reference. The length of the duplexes was varied between 20 and 25 bp by extending the base-paired segment at the 3′ end of the sense siRNA (Figure 3B) or at the 3′ end of the antisense siRNA (Figure 3C).

Duplexes of 20–23 bp caused specific repression of target luciferase activity, but the 21 nt siRNA duplex was at least 8-fold more efficient than any of the other duplexes. siRNA duplexes of 24 and 25 nt did not result in any detectable interference. Sequence-specific effects were minor as variations on both ends of the duplex produced similar effects.

2′-deoxy- and 2′-O-methyl-modified siRNA duplexes

To assess the importance of the siRNA ribose residues for RNAi, duplexes with 21 nt siRNAs and 2 nt 3′ overhangs with 2′-deoxy- or 2′-*O*-methyl-modified strands were examined (Figure 4). Substitution of the 2 nt 3′ overhangs by 2′-deoxynucleotides had no effect and even the replacement of two additional ribonucleotides by 2′-deoxyribonucleotides adjacent to the overhangs in the paired region produced significantly active siRNAs. Thus, 8 out of 42 nt of a siRNA duplex were replaced by DNA

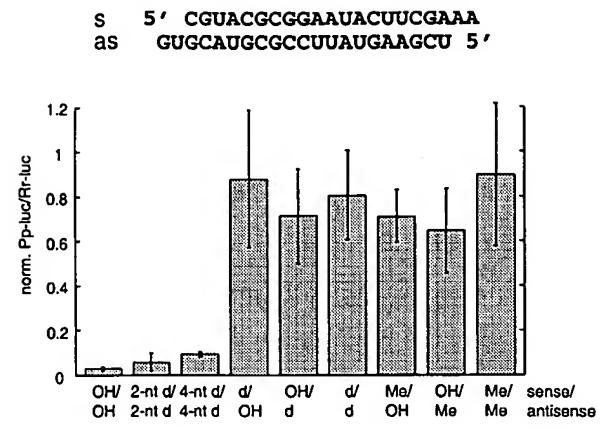


Fig. 4. Substitution of the 2′-hydroxyl groups of the siRNA ribose residues. The 2′-hydroxyl groups (OH) in the strands of siRNA duplexes were replaced by 2′-deoxy (d) or 2′-*O*-methyl (Me). 2 and 4 nt 2′-deoxy substitutions at the 3′ ends are indicated as 2- and 4-nt d, respectively. Uridine residues were replaced by 2′-deoxythymidine.

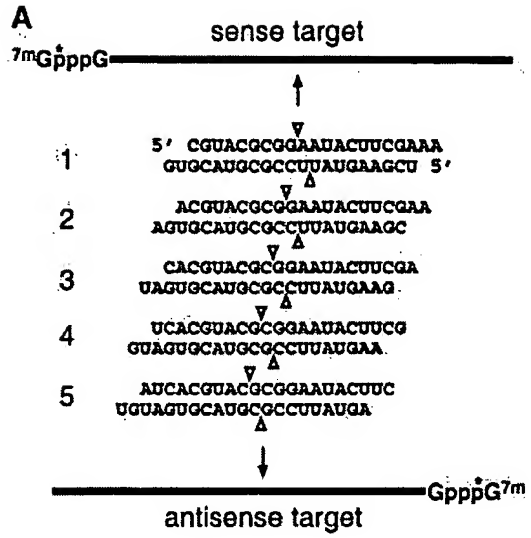
residues without loss of activity. Complete substitution of one or both siRNA strands by 2′-deoxy residues, however, abolished RNAi, as did complete substitution by 2′-*O*-methyl residues.

Definition of target RNA cleavage sites

Target RNA cleavage positions were previously determined for 22 nt siRNA duplexes and for a 21 and 22 nt duplex (Elbashir *et al.*, 2001b). The position of target RNA cleavage was located in the centre of the region covered by the siRNA duplex, 11 or 12 nt downstream of the first nucleotide that was complementary to the 21 or 22 nt siRNA guide sequence. Five distinct 21 nt siRNA duplexes with 2 nt 3′ overhang (Figure 5A) were incubated with 5′ cap-labelled sense or antisense target RNA in *D.melanogaster* lysate (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). The 5′ cleavage products were resolved on sequencing gels (Figure 5B). The amount of sense target RNA cleaved correlated with the efficiency of siRNA duplexes determined in the translation-based assay, and siRNA duplexes 1, 2 and 4 (Figures 5B, 1E, G and H) cleaved target RNA faster than duplexes 3 and 5 (Figures 5B, 1D and F). Notably, the sum of radioactivity of the 5′ cleavage product and the input target RNA were not constant over time and the 5′ cleavage products did not accumulate. Presumably, the cleavage products, once released from the siRNA–endonuclease complex, were rapidly degraded due to the lack of either the poly(A) tail or the 5′ cap.

The cleavage sites for both sense and antisense target RNAs were located in the middle of the region spanned by the siRNA duplexes. The cleavage sites for each target produced by the five different duplexes varied by 1 nt according to the 1 nt displacement of the duplexes along the target sequences. The targets were cleaved precisely 11 nt downstream of the target position complementary to the 3′-most nucleotide of the sequence-complementary guide siRNA (Figure 5).

In order to determine whether the 5′ or the 3′ end of the guide siRNA sets the ruler for target RNA cleavage, we devised the experimental strategy outlined in Figure 6A



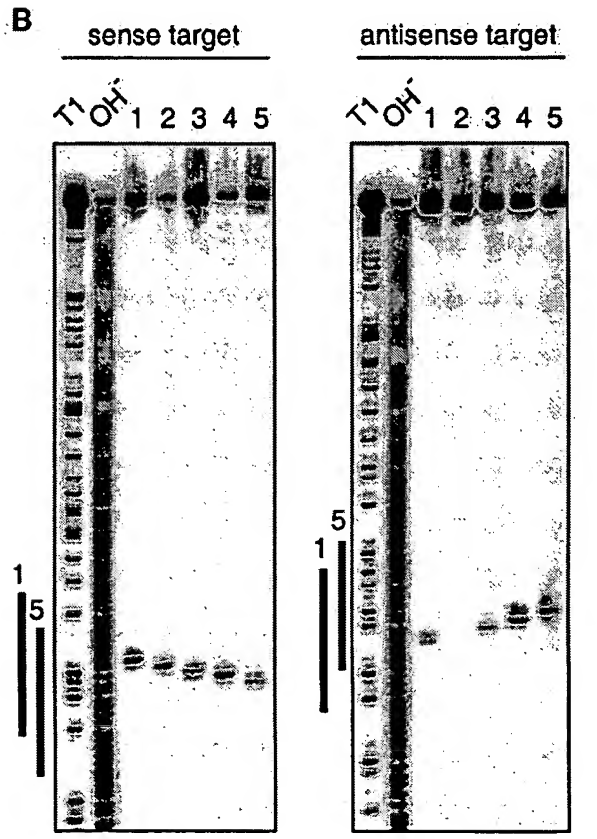


Fig. 5. Mapping of sense and antisense target RNA cleavage by 21 nt siRNA duplexes with 2 nt 3′ overhangs. (A) Representation of ^{32}P (asterisk) cap-labelled sense and antisense target RNAs and siRNA duplexes. The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (B) Mapping of target RNA cleavage sites. After 2 h incubation of 10 nM target RNA with 100 nM siRNA duplex in *D.melanogaster* embryo lysate, the 5′ cap-labelled substrate and the 5′ cleavage products were resolved on 6% sequencing gels. Length markers were generated by partial RNase T1 digestion (T1) and partial alkaline hydrolysis (OH) of the target RNAs. The bold lines to the left of the images indicate the region covered by the siRNA strands 1 and 5 of the same orientation as the target.

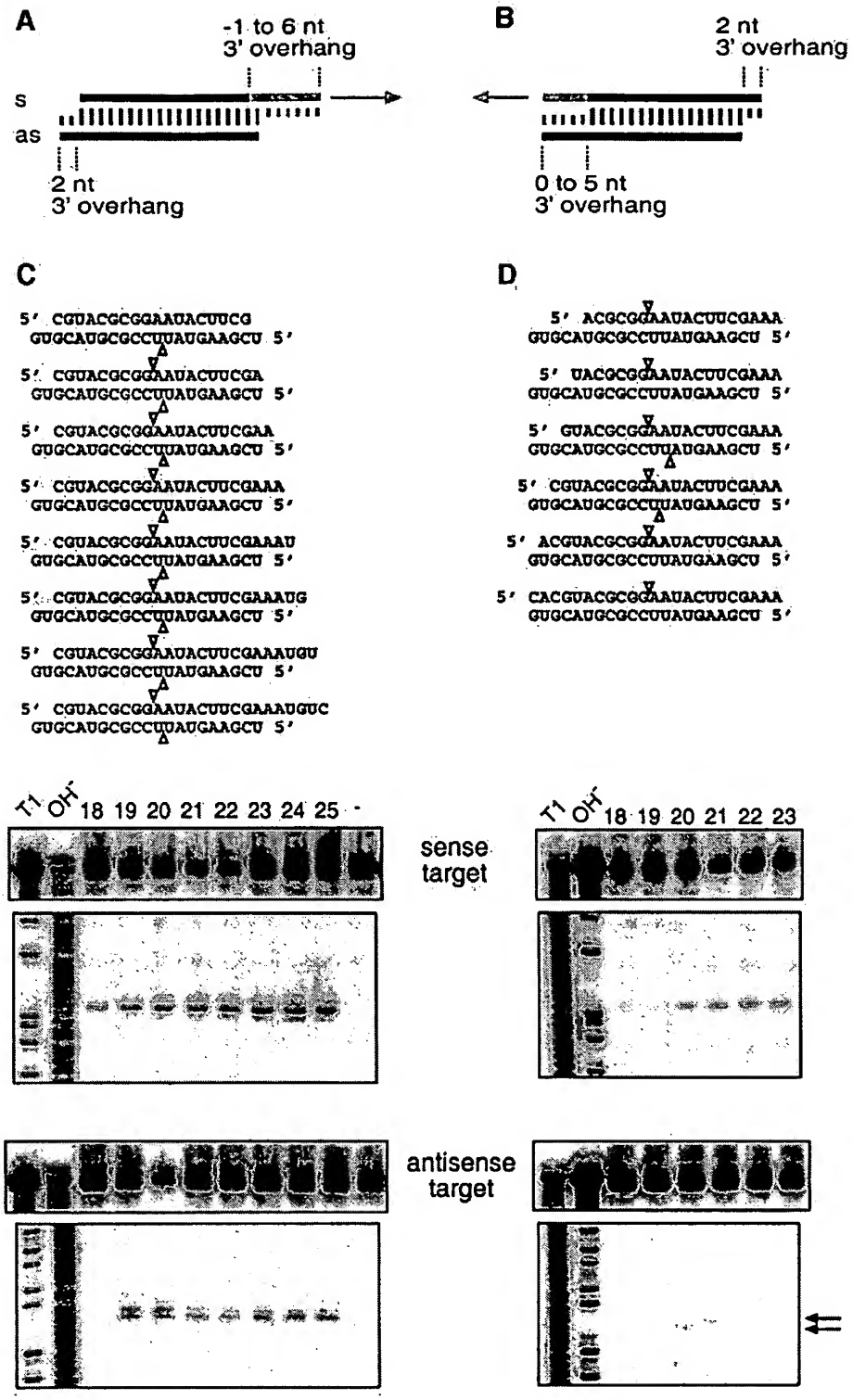


Fig. 6. The 5′ end of a guide siRNA defines the position of target RNA cleavage. (A and B) Representation of the experimental strategy. The antisense siRNA was the same in all siRNA duplexes, but the sense strand was varied between 18 and 25 nt by changing the 3′ end (A) or 18 and 23 nt by changing the 5′ end (B). The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (C and D) Analysis of target RNA cleavage using cap-labelled sense (top) or antisense (bottom) target RNAs. The residual amount of targeted substrate and the cap-labelled 5′ cleavage products are shown. The sequences of the siRNA duplexes are indicated and the length of the sense siRNA strands is marked on top. The control lane, marked with a dash in (C), shows target RNA incubated in absence of siRNAs. Markers were as described in Figure 5. The arrows in (D), bottom, indicate the target RNA cleavage sites that differ by 1 nt.

and B. A 21 nt antisense siRNA, which was kept invariant for this study, was paired with sense siRNAs that were modified in length at either of their 5′ or 3′ ends. The position of sense and antisense target RNA cleavage was

determined as described above. Changes in the 3′ end of the sense siRNA, monitored for 1 nt 5′ overhang to 6 nt 3′ overhang, did not affect either the position of sense nor antisense target RNA cleavage (Figure 6C). Changes in the 5′ end of the sense siRNA did not affect the sense target RNA cleavage (Figure 6D, top), as expected, because the antisense siRNA was unchanged. The residual amount of uncleaved sense target RNA (Figure 6C and D top) correlated with the efficiency of siRNA duplexes determined in translation-based assays (Figure 2C and data not shown), but did not correlate with the amount of detected cleavage product. Accumulation of cleavage products was more pronounced for the longer and less efficient siRNA duplexes, suggesting that product release may have become rate limiting. Because the antisense siRNA was kept unchanged while the sense siRNA was varied, an alteration in product release implies a role of the sense siRNA strand in the target RNA degradation process.

Changes in the 5′ end of the sense siRNA, in contrast to its 3′ end, strongly affected antisense target RNA cleavage (Figure 6D, bottom). The antisense target was only cleaved when the sense siRNA was 20 or 21 nt in size. The position of cleavage differed by 1 nt, suggesting that the 5′ end of the target-recognizing siRNA sets the ruler for target RNA cleavage. This position is located between nucleotide 10 and 11 when counting in an upstream direction from the target nucleotide paired to the 5′-most nucleotide of the guide siRNA (see also Figure 5A).

Sequence effects and 2′-deoxy substitutions in the 3′ overhang

The 2 nt 3′ overhang is critical for siRNA function. We wanted to know whether the sequence of the overhanging nucleotides contributes to target recognition or is only a feature required for reconstitution of the endonuclease complex (RISC or siRNP). We synthesized sense and antisense siRNAs with AA, CC, GG, UU and UG 3′ overhangs and included the 2′-deoxy modifications TdG and TT (T, 2′-deoxythymidine; dG, 2′-deoxyguanosine). The wild-type siRNAs contained AA in the sense 3′ overhang and UG in the antisense 3′ overhang (AA/UG). All siRNA duplexes were functional in the interference assay and reduced target expression at least 5-fold (Figure 7). The most efficient siRNA duplexes, which reduced target expression >10-fold, were of the sequence type NN/UG, NN/UU, NN/TdG and NN/TT (N, any nucleotide). siRNA duplexes with an antisense siRNA 3′ overhang of AA, CC or GG were less active by a factor of 2–4 when compared with the wild-type sequence UG or the mutant UU. This reduction in RNAi efficiency is likely to be due to the contribution of the penultimate 3′ nucleotide to sequence-specific target recognition, as the 3′-terminal nucleotide was changed from G to U without effect.

Changes in the sequence of the 3′ overhang of the sense siRNA did not reveal any sequence-dependent effects, which was not surprising because the sense siRNA is not expected to contribute to the sequence-specific recognition of the sense target mRNA.

Sequence specificity of target recognition

In order to examine the sequence specificity of target recognition, we introduced sequence changes into the

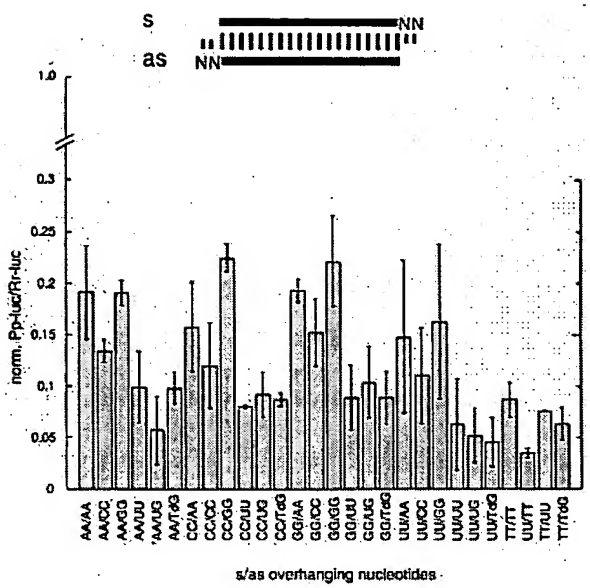


Fig. 7. Sequence variation of the 3′ overhang of siRNA duplexes. The 2 nt 3′ overhang (NN, in grey) was changed in sequence and composition as indicated (T, 2′-deoxythymidine; dG, 2′-deoxyguanosine; asterisk, wild-type siRNA duplex). Normalized interference ratios were determined as described in Figure 1. The wild-type sequence is the same as depicted in Figure 4.

paired segments of siRNA duplexes and determined the efficiency of silencing. Sequence changes were introduced by inverting short segments of 3 or 4 nt or inducing point mutations (Figure 8). The sequence changes in one siRNA strand were compensated for in the complementary siRNA strand to avoid perturbing the base-paired siRNA duplex structure. The sequence of all 2 nt 3′ overhangs was TT to reduce costs of synthesis. The TT/TT reference siRNA duplex was comparable in RNAi to the wild-type siRNA duplex AA/UG (Figure 7). The ability to mediate reporter mRNA destruction was quantified using the translation-based luminescence assay. Duplexes of siRNAs with inverted sequence segments showed dramatically reduced ability for targeting the firefly luciferase reporter (Figure 8). The sequence changes located between the 3′ end and the middle of the antisense siRNA completely abolished target RNA recognition, but mutations near the 5′ end of the antisense siRNA exhibited a small degree of silencing. Transversion of the AU base pair located directly opposite the predicted target RNA cleavage site or 1 nt further away from the predicted site prevented target RNA cleavage, therefore indicating that a single mutation within the centre of a siRNA duplex discriminates between mismatched targets.

Discussion

siRNAs are valuable reagents for inactivation of gene expression, not only in insect cells but also in mammalian cells, with a great potential for therapeutic application (Elbashir *et al.*, 2001a). We have systematically analysed the structural determinants of siRNA duplexes required to promote efficient target RNA degradation in *D.melanogaster* embryo lysate, thus providing rules for the design of most potent siRNA duplexes. A perfect siRNA duplex is able to silence gene expression with an

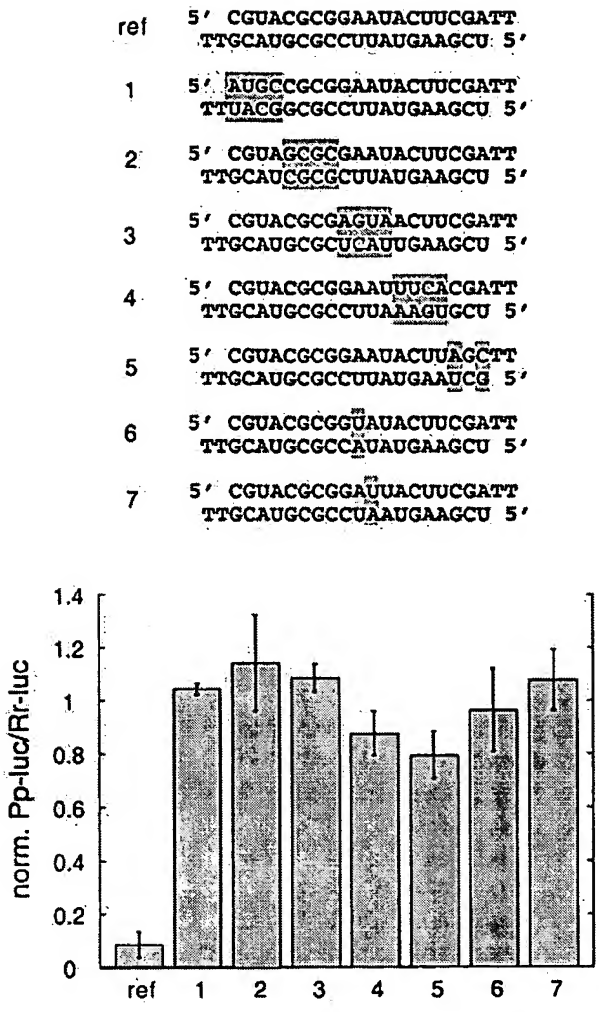


Fig. 8. Sequence specificity of target recognition. The sequences of the mismatched siRNA duplexes are shown, modified sequence segments or single nucleotides are shaded in grey. The reference duplex (ref) and the siRNA duplexes 1–7 contain 2′-deoxythymidine 2 nt overhangs. The silencing efficiency of the thymidine-modified reference duplex was comparable to the wild-type sequence (Figure 7). Normalized interference ratios were determined as described in Figure 1.

efficiency comparable to a 500 bp dsRNA, given that comparable quantities of total RNA are used.

The siRNA user guide

Efficiently silencing siRNA duplexes are composed of 21 nt sense and 21 nt antisense siRNAs and must be selected to form a 19 bp double helix with 2 nt 3′-overhanging ends. 2′-deoxy substitutions of the 2 nt 3′-overhanging ribonucleotides do not affect RNAi, but help to reduce the costs of RNA synthesis and may enhance RNase resistance of siRNA duplexes. More extensive 2′-deoxy or 2′-*O*-methyl modifications reduce the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNP assembly.

Target recognition is a highly sequence-specific process, mediated by the siRNA complementary to the target. The 3′-most nucleotide of the guide siRNA does not contribute to the specificity of target recognition, while the penultimate nucleotide of the 3′ overhang affects target RNA cleavage and a mismatch reduces RNAi 2- to 4-fold. The 5′ end of the guide siRNA also appears more permissive for mismatched target RNA recognition when compared with the 3′ end. Nucleotides in the centre of the siRNA, located opposite to the target RNA cleavage site, are important specificity determinants and even single nucleotide changes reduce RNAi to undetectable levels. This suggests that siRNA duplexes may be able to discriminate mutant or polymorphic alleles in gene targeting experiments, which may become an important feature for future therapeutic developments.

Sense and antisense siRNAs, when associated with the protein components of the endonuclease complex or its commitment complex, were suggested to play distinct roles; the relative orientation of the siRNA duplex in this complex defines which strand can be used for target recognition (Elbashir *et al.*, 2001b). Synthetic siRNA duplexes with an equal number of overhanging nucleotides have dyad symmetry with respect to the double-helical structure, but not with respect to sequence. The association of siRNA duplexes with the RNAi proteins in the *D.melanogaster* lysate leads to the formation of two asymmetric complexes. In such hypothetical complexes, the chiral environment is distinct for sense and antisense siRNA, hence their function. The prediction obviously does not apply to palindromic siRNA sequences or to RNAi proteins that could associate as homodimers. To minimize sequence effects that may affect the ratio of sense- and antisense-targeting siRNPs, we suggest using siRNA sequences with identical 3′-overhanging sequences. We recommend adjusting the sequence of the overhang of the sense siRNA to that of the antisense 3′ overhang because the sense siRNA does not have a target in typical knock-down experiments. Asymmetry in the reconstitution of sense- and antisense-cleaving siRNPs could be, partially, responsible for the variation in RNAi efficiency observed for various 21 nt siRNA duplexes with 2 nt 3′ overhangs used in this study (Figure 1). Alternatively, the nucleotide sequence at the target site and/or the accessibility of the target RNA structure may be responsible for the variation in efficiency observed for these siRNA duplexes. It should be noted that all siRNAs used in this study are derived from a short region of one gene. Thus, it is more likely that differences in siRNA efficiency are a consequence of the primary sequences of the siRNAs and the respective target sites, rather than the secondary or tertiary structure of the targeted RNA.

Natural siRNAs versus synthetic siRNAs

In *D.melanogaster*, siRNA duplexes are produced *in vitro* and *in vivo* from long dsRNAs (Hammond *et al.*, 2000; Yang *et al.*, 2000; Zamore *et al.*, 2000). About 45% of these short RNAs are precisely 21 nt long, 28% are 22 nt long and a few percent are shorter or longer RNAs (Elbashir *et al.*, 2001b). This length distribution correlates with our finding that 21 nt siRNA duplexes are the most efficient mediators of mRNA degradation. Beside the length, the paired structure and overhang are also important. This structural feature may explain why siRNA duplexes isolated from the dsRNA processing reaction under denaturing conditions were less potent for RNAi than longer dsRNAs that were processed to siRNAs during the targeting reaction (Zamore *et al.*, 2000). Presumably,

denaturation followed by renaturation favoured the formation of the thermodynamically more stable, blunt-ended, but less active, siRNA duplexes. Isolation of siRNAs under native conditions does not reduce siRNA activity (Nykänen *et al.*, 2001).

Production of siRNAs from long dsRNA requires the RNase III enzyme dicer (Bernstein *et al.*, 2001). Dicer is a bidentate RNase III, which also contains an ATP-dependent RNA helicase domain and a PAZ domain, presumably important for dsRNA unwinding and mediation of protein–protein interactions, respectively (Cerutti *et al.*, 2000; Bernstein *et al.*, 2001). Dicer is evolutionarily conserved in worms, flies, plants, fungi and mammals (Matsuda *et al.*, 2000), and has a second cellular function important for the development of these organisms (Ray *et al.*, 1996; Jacobsen *et al.*, 1999; Grishok *et al.*, 2001; Hutvágner *et al.*, 2001; Knight and Bass, 2001). At present, it is uncertain whether dicer activity in species other than *D.melanogaster* produces siRNAs of predominantly 21 nt in length. The estimates of siRNA size vary in the literature between 21 and 25 nt (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Hutvágner *et al.*, 2000; Parrish *et al.*, 2000; Yang *et al.*, 2000; Zamore *et al.*, 2000; Elbashir *et al.*, 2001b).

In a recent study of the effect of siRNA length in mammalian cells (primary mouse embryonic fibroblasts, 293 and HeLa cells), duplexes of 21–27 nt siRNAs with 2 nt 3′ overhangs were directed against different co-transfected reporter genes (Caplen *et al.*, 2001). Duplexes of 22 and 23 nt siRNAs were found to be slightly more efficient in triggering sequence-specific gene silencing than 21 nt siRNA duplexes. In our hands, using the dual luciferase assay system in HeLa cells, 21 nt siRNA duplexes with 2 nt 3′ overhang are 2- to 3-fold more efficient than 20 or 22–25 nt siRNA duplexes (data not shown), therefore recapitulating the results obtained from the *D.melanogaster* biochemical system. In contrast to the *D.melanogaster* system, siRNA duplexes >23 nt in length are still triggering some RNAi in HeLa cells and also in *C.elegans* (Caplen *et al.*, 2001). However, it remains to be determined whether the RNA strands finally incorporated into the active endonuclease complex are of the initially provided length. It is possible that exonucleases present in *C.elegans* and mammalian cells trim longer siRNAs to their optimum length and that these exonucleases are absent from *D.melanogaster* lysate.

The functional anatomy of long dsRNAs as a trigger for RNAi was analysed previously in *C.elegans* (Parrish *et al.*, 2000). Activation of RNAi by injection of long dsRNA requires at least two steps: dsRNA processing by dicer RNase III and siRNP or RISC formation. Substitution of one of the strands of the long dsRNA by DNA abolished RNAi and even the substitution of C by dC or U by dT in only one of the strands caused a substantial decrease in RNAi. Because introduction of 2′-fluoro modifications into long RNA had no effect, it was suggested that an A-form double helical structure was important for triggering RNAi (Parrish *et al.*, 2000). We have been able to substitute eight ribose residues of a siRNA duplex by 2′-deoxyribose residues without substantial reduction of RNAi, although it should be noted the 2′-deoxy modifications were clustered at the 3′ end of the siRNAs, including the 2 nt 3′ overhangs. It is possible that the four 2′-deoxy modifications, which are located in the paired region at the end of the helix, do not affect the overall A-form helical structure and do not strongly compromise RISC formation. Complete modification of one or both siRNA strands by 2′-deoxyribose, however, abolished RNAi. Interestingly, substitution by 2′-*O*-methylribose, which adopts the ribose sugar pucker, also abolished RNAi, probably because methylation of the 2′-hydroxyls blocked hydrogen bond formation or introduced steric hindrance.

It was recently demonstrated that a 5′-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA function and that ATP is used to maintain the 5′-phosphates of the siRNAs (Nykänen *et al.*, 2001). However, 5′-phosphorylation of fully 2′-deoxy- or 2′-*O*-methyl-modified siRNA strands was not able to restore RNAi (data not shown). Unmodified siRNA duplexes with free 5′-hydroxyls and 2 nt 3′ overhangs are readily phosphorylated in *D.melanogaster* embryo lysate (Nykänen *et al.*, 2001). In this respect, it should be noted that our reported RNAi efficiencies were determined by pre-incubating the siRNA duplexes for 15 min in *D.melanogaster* lysate before adding target and control mRNAs, thus providing sufficient time for 5′-phosphorylation of siRNA duplexes to occur. Comparison of the RNAi efficiencies of 5′-phosphorylated and 5′-non-phosphorylated siRNAs (for duplexes shown in Figures 1E, F and 2C) did not reveal any sizeable differences (data not shown).

Conclusions

We have performed an extensive analysis of the length, sequence and structure of siRNA duplexes in *D.melanogaster* embryo lysate. Duplexes of 21 nt siRNAs with 2 nt 3′ overhangs were shown to be the most efficient triggers of RNAi-based mRNA degradation. The target recognition is a highly sequence-specific process, although not all positions of a guide siRNA contribute equally to specificity. These results are important for the design of efficient siRNAs in order to silence genes in *D.melanogaster* and provide a basis for similar studies in other organisms.

Materials and methods

RNA preparation and RNAi assay

Chemical RNA synthesis, annealing and luciferase-based RNAi assays were performed as described previously (Tuschl *et al.*, 1999; Zamore *et al.*, 2000; Elbashir *et al.*, 2001b). Synthetic RNAs were gel purified after deprotection. The formation of siRNA duplexes was verified by agarose gel electrophoresis using 4% NuSieve GTG agarose (BMA, Rockland, ME) in 0.5× TBE buffer. All siRNA duplexes were directed against firefly luciferase and the luciferase mRNA sequence was derived from pGEM-luc (DDBJ/EMBL/GenBank accession No. X65316) as described (Tuschl *et al.*, 1999). The siRNA duplexes were incubated in a *D.melanogaster* RNAi/translation reaction for 15 min prior to addition of mRNAs. Translation-based RNAi assays were performed at least in triplicate.

For mapping of sense target RNA cleavage, a 177 nt transcript was generated, corresponding to the firefly luciferase sequence between positions 113 and 273 relative to the start codon, followed by the 17 nt complement of the SP6 promoter sequence (Elbashir *et al.*, 2001b). For mapping of antisense target RNA cleavage, a 166 nt transcript was produced from a template, which was amplified from plasmid sequence by PCR using the 5′ primer TAATACGACTCACTATAGAGCCCATATCGTTTCATA (T7 promoter underlined) and 3′ primer AGAG-

GATGGAACCGCTGG. The target sequence corresponds to the complement of the firefly luciferase sequence between positions 50 and 215 relative to the start codon. Guanylyl transferase labelling was performed as described previously (Zamore *et al.*, 2000). For mapping of target RNA cleavage, 100 nM siRNA duplex was incubated with 5–10 nM target RNA in *D.melanogaster* embryo lysate under standard conditions (Zamore *et al.*, 2000) for 2 h at 25°C. The reaction was stopped by the addition of 8 vols of proteinase K buffer [200 mM Tris–HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (w/v) SDS]. Proteinase K (dissolved in water; Merck) was added to a final concentration of 0.6 mg/ml. The reactions were then incubated for 15 min at 65°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 3 vols of ethanol. Samples were loaded on 6% sequencing gels. Length standards were generated by partial RNase T1 digestion and partial base hydrolysis of the cap-labelled sense or antisense target RNAs.

Acknowledgements

We acknowledge Heike Taubner and Jutta Meyer for technical assistance, Phil Zamore for critical comments on the manuscript and H.Jäckle and R.Lührmann for support. This work was funded by a BMBF Biofuture grant, No. 0311856.

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Received August 23, 2001; revised October 10, 2001; accepted October 16, 2001

EXHIBIT 3 of

EXHIBIT B

Nucleic Acids Research, 2003, Vol. 31, No. 2 589–595
DOI: 10.1093/nar/gkg147

Tolerance for mutations and chemical modifications in a siRNA

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Received October 9, 2002; Revised and Accepted November 12, 2002

ABSTRACT

Short interfering RNA (siRNA), the active agent of RNA interference, shows promise of becoming a valuable tool in both basic and clinical research. We explore the tolerance to mutations and chemical modifications in various parts of the two 21-nt strands of a siRNA targeting the blood clotting initiator Tissue Factor. The mutations were G/C transversions. The chemical modifications were 2′-*O*-methylation, 2′-*O*-allylation and phosphorothioates. We found that siRNA generally tolerated mutations in the 5′ end, while the 3′ end exhibited low tolerance. This observation may facilitate the design of siRNA for specific targeting of transcripts containing single nucleotide polymorphisms. We further demonstrate that in our system the single antisense strand of the wild-type siRNA is almost as effective as the siRNA duplex, while the corresponding methylated M2+4 version of the antisense had reduced activity. Most of the chemically modified versions tested had near-wild-type initial activity, while the long-term activity was increased for certain siRNA species. Our results may improve the design of siRNAs for *in vivo* experiments.

INTRODUCTION

RNA interference (RNAi), the process of depleting RNA targets by the use of double-stranded RNA (dsRNA), was first reported in 1998 (1). Since the demonstration of the efficacy of short interfering RNA (siRNA) in mammalian cells (2–4), this new tool has been used to successfully target various infectious agents like HIV, poliovirus and RSV (5–9). In some cases, newly constructed vectors were used to produce short hairpin RNA (shRNA) that is processed to siRNA intracellularly (10–14).

The mechanism of RNAi is not well understood (15–22). The triggering long dsRNA or aberrant RNA is assumed to be processed by the RNase III-like enzyme Dicer to approximately 21–23 nt siRNA, which is then incorporated into the RNA-induced silencing complex (RISC). This step can be bypassed by transfection of chemically synthesised siRNA. Dicer has also been implicated in processing of the siRNA-related short temporal RNA let-7, shown in *Caenorhabditis elegans* to be involved in control of larval development, and widely conserved in other species, among them humans (23–25).

The physiological function of RNAi is assumed to be defence against viral infections. This has been convincingly demonstrated in plants, where some viruses have evolved counter-measures against RNAi (26,27). An insect virus has recently been shown to both activate RNA silencing and express a suppressor protein in *Drosophila* cells (28). In *C.elegans*, mutations in RNAi genes have resulted in the activation of transposons (29,30), arguing for their involvement in the defence against these genomic parasites.

The tolerance of the effect of siRNA for mutations is still unclear. Boutla *et al.* (31) reported that a mutated siRNA with a single centrally located mismatch relative to the mRNA target sequence retained substantial activity in *Drosophila*. In contrast, Elbashir *et al.* (32) found that a single mismatch was deleterious to activity in an *in vitro Drosophila* embryo lysate assay. In previous work (33) we tried to reconcile these conflicting results by depicting the RNAi process *in vivo* as a dynamic one where several factors influenced the final outcome, among them siRNA target position, siRNA concentration, mRNA concentration, mRNA synthesis rate and siRNA's inherent cleavage activity, an activity that can be reduced gradually by mismatch mutations.

In the present work we explore how various mutations and chemical modifications alter the efficacy and duration of our most effective siRNA (hTF167i) targeting the human Tissue Factor (hTF) mRNA. The objectives were, firstly, to find regions less tolerant in their siRNA effect for single mutations, thus possibly facilitating the design of siRNA for specific targeting of transcripts containing single nucleotide polymorphisms. Secondly, we wished to improve the long-term activity of our siRNA by stabilising the RNA strands against nucleases through introducing phosphorothioates and modifications of the 2′-OH.

We find that hTF167i has a general tolerance to mutations, with less tolerance for mutations at 3′ end of the siRNA. Furthermore, with the exception of certain allyl-modifications, the backbone modifications did not reduce the activity of the siRNA to a significant degree. Extensive use of phosphorothioate modifications resulted in cytotoxicity. The 2′-*O*-methylation modifications, on the other hand, showed promise

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

as they resulted in increased persistence of activity with no toxicity to cells.

MATERIALS AND METHODS

SiRNA preparation

RNAs (21 nt) were chemically synthesised using phosphoramidites (Glen Research and PerSeptive Biosystems). Deprotected and desilylated synthetic oligoribonucleotides were purified by reverse-phase HPLC. Ribonucleotides were annealed at 10 μM in 200 μl 10 mM Tris–HCl pH 7.5 by boiling and gradual cooling in a water bath. Successful annealing was confirmed by non-denaturing (15%) polyacrylamide gel electrophoresis.

Cell culture and transfections

The human keratinocyte cell line HaCaT was cultured in serum-free keratinocyte medium (Gibco BRL) supplemented with 2.5 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract. The cell line was regularly passaged at sub-confluence and plated 1 or 2 days before transfection. HaCaT cells in 6-well plates were transfected at low confluency (<40%) with 1.0 ml 100 nM siRNA in serum-free medium, using Lipofectamine 2000. For complexation, 10 μM stock solution of siRNA was diluted with 10× vol. of serum-free medium and mixed with an equal volume of medium-diluted Lipofectamine 2000, at a v/w ratio of liposome to siRNA of 5:2. Batch dilutions of liposomes were performed for each 6-well plate and allowed to pre-incubate at room temperature for 5–7 min before addition to the medium-diluted siRNA. Complexes were replaced with full medium 5 h after initiation of transfection. For standard assays of activity, cells were harvested the day after transfection. For longer incubations and time-course experiments, medium was replaced every second day after transfection.

Northern analysis

Polyadenylated mRNA was isolated using Dynabeads oligo(dT)$_{25}$ (Dynal). Isolated mRNA was fractionated by electrophoresis for 16–18 h on 1.3% agarose/formaldehyde (0.8 M) gels and blotted on to nylon membranes (MagnaCharge, Micron Separations). Membranes were hybridised with random-primed Tissue Factor (TF) (position 61–1217 in cDNA) and GAPDH (1.2 kb) cDNA probes in PerfectHyb hybridisation buffer (Sigma).

RESULTS

Mutational scanning of siRNA targeting hTF167

We have previously demonstrated that one and two central mutations in siRNA targeting position 167 in hTF did not abolish its ability to deplete endogenous TF mRNA levels (33). We decided to map this siRNA more systematically in order to determine whether mutations were tolerated equally well within the whole siRNA. To simplify analysis, only GC pairs were mutated by inversion of the pairs. A total of eight different single-mutant siRNAs were designed and named according to the position (starting from the 5′ of the sense strand) of the mutation (s1, s2, s3, s4, s7, s11, s13, s16) (Fig. 1).

wt	5′-gcgcuucaggcacuacaaaua-3′ 3′-gccgcgaaguccgugauguuu-5′
s1	5′-**c**cgcuucaggcacuacaaaua-3′ 3′-gc**g**gcgaaguccgugauguuu-5′
s2	5′-g**g**gcuucaggcacuacaaaua-3′ 3′-gc**c**ccgaaguccgugauguuu-5′
s3	5′-gcc**c**uucaggcacuacaaaua-3′ 3′-gcc**g**ggaaguccgugauguuu-5′
s4	5′-gcg**g**uucaggcacuacaaaua-3′ 3′-gcc**c**gcaaguccgugauguuu-5′
s7	5′-gcgcuu**g**aggcacuacaaaua-3′ 3′-gccgcgaa**c**uccgugauguuu-5′
s10	5′-gcgcuucag**c**cacuacaaaua-3′ 3′-gccgcgaaguc**g**gugauguuu-5′
s11	5′-gcgcuucagg**g**acuacaaaua-3′ 3′-gccgcgaaguccc**u**gauguuu-5′
s13	5′-gcgcuucaggc**a**uacaaaua-3′ 3′-gccgcgaaguccg**u**cauguuu-5′
s16	5′-gcgcuucaggcacua**g**aaaua-3′ 3′-gccgcgaaguccgugau**c**uuu-5′
ds7/10	5′-gcgcuu**g**agc**c**acuacaaaua-3′ 3′-gccgcgaa**c**uc**g**gugauguuu-5′
ds10/11	5′-gcgcuucag**c**g**a**cuacaaaua-3′ 3′-gccgcgaagu**c**g**c**ugauguuu-5′
ds10/13	5′-gcgcuucag**c**c**a**guacaaaua-3′ 3′-gccgcgaagu**c**gg**u**cauguuu-5′
ds10/16	5′-gcgcuucag**c**cacua**g**aaaua-3′ 3′-gccgcgaagu**c**ggugau**c**uuu-5′

Figure 1. Mutated and wild-type (wt) versions of the siRNA hTF167i. The sequence of the sense strand of wild-type siRNA corresponds to position 167–187 in hTF (Acc.No. M16553). Single (s1, s2, s3, s4, s7, s10, s11, s13, s16) and double (ds7/10, ds10/11, ds10/13, ds10/16) mutants are all named according to the position of the mutation, counted from the 5′ end of the sense strand. All mutations (in bold) are GC inversions relative to the wild-type.

The previously described centrally located single-mutant M1 (33), was included in this analysis and renamed s10.

These mutant siRNAs were analysed for their ability to deplete endogenous TF mRNA in HaCaT cells (Fig. 2). The wild-type siRNA, and the mutant s10, included as positive controls, depleted TF mRNA to 10 and 20% residual levels, as reported previously (33). The activities of the other mutants could be categorised into three groups depending on their position along the siRNA. Mutations in the extreme 5′ end of the siRNA were well tolerated, exhibiting essentially the same activity as the wild-type. Mutations localised towards the 3′ end, and up to the approximate midpoint of the siRNA (s4, s7, s10, s11), were slightly impaired in their activity, resulting in depletion of mRNA to 20–30% residual levels. Both of the mutations in the 3′ half of the siRNA (s13, s16), however, exhibited severely impaired activity, suggesting a bias in the tolerance for mutations in the reaction(s) involving siRNA. The activities of four double mutants, in which the central position (s10) was mutated in conjunction with one additional position (s7, s11, s13,. s16), were also analysed. The bias in mutation tolerance was also evident for these double mutants, as the rank order of their activity mirrored that of the activity

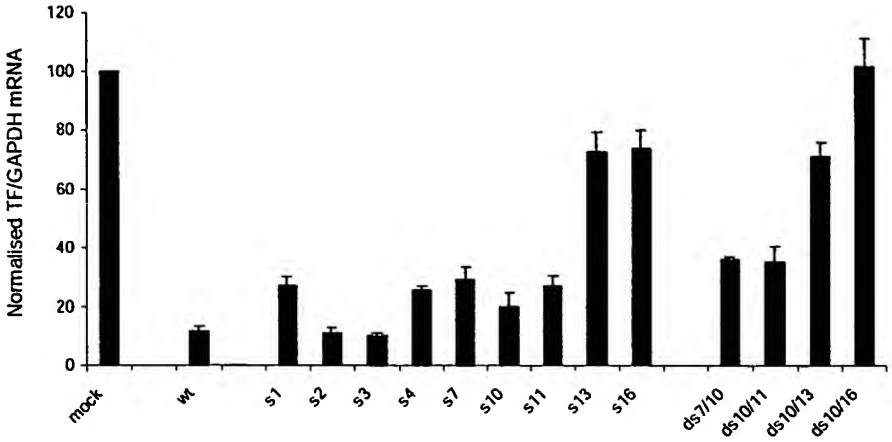

Figure 2. Activity of mutant siRNAs against endogenous hTF mRNA. HaCaT cells were harvested for mRNA isolation 24 h post-transfection. TF expression was normalised to that of GAPDH. Normalised expression in mock-transfected cells was set as 100%. Data are averages + s.d. of at least three independent experiments.

of the single mutants of the variant position. This observation strengthens the proposition that the differential activity of mutants is due to an intrinsic bias in the tolerance for target mismatches along the sequence of the siRNA.

Chemical modification of siRNAs for increased persistence of silencing

The effect of siRNA in human cells is transient and typically decreases during a few days in cell culture (18,33). The ability to extend the period of effective silencing would be of importance for the possible use of siRNA in therapy. To increase the intracellular stability of siRNA, we introduced a gradual increase of various chemical modifications to both ends of the siRNA. We have previously used phosphorothioate linkages and 2′-*O*-modifications in the form of methylation and allylation to stabilise hammerhead ribozymes (34), and decided to try a similar strategy for siRNAs. In all modified siRNA species, the same modifications were introduced in both strands. It has been reported that siRNA with a general 2′-*O*-methylation in either strand have no activity (32). We therefore started with less extensive modification. Initially, siRNAs with one modification in each of the extreme 5′ and 3′ ends of their strands (P1+1, M1+1, A1+1, Fig. 3) were synthesised. The 5′ end of the siRNAs might be more sensitive to modification since it must be phosphorylated in order to be active *in vivo* (22). We therefore included siRNAs with modifications only in the non-basepairing 3′ overhangs (P0+2, M0+2 and A0+2, Fig. 3), which were known to tolerate various types of modifications (2,3,32,33).

Northern analysis of transfected HaCaT cells demonstrated essentially undiminished activity of all the chemically modified siRNAs, with the exception of the siRNA with allylation at both ends (Fig. 3). Allyl-modification in the 3′ end only had no effect on activity. The presence of a bulky substituent in the 2′-hydroxyl of the 5′ terminal nucleotide might interfere with the *in vivo* phosphorylation of the siRNA necessary for its activity (22). Further modification of the siRNA by methylation resulted in gradual attenuation of activity. One day after transfection with siRNAs carrying 4+4, 4+6, 6+6 or 6+8 methyl groups in their ends, the cells exhibited residual reporter gene expression levels of 28, 42, 68 and 75%, respectively.

We next wanted to know if any of these modifications increased the persistence of siRNA-mediated silencing. The level of endogenous TF mRNA recovers gradually 3–5 days after transfection with wild-type siRNA targeting hTF167 (33). Transfecting HaCaT cells with unmodified and chemically modified siRNAs in parallel did not result in any significant differences in their silencing activities at 3 and 5 days post-transfection until a rather high degree of modification was used. Allylated siRNAs were not tested in this experiment, since they showed reduced effectivity even with only one substituent in the 5′ end (Fig. 3). SiRNAs methylated in the 2′-OH in up to 2+4 positions showed well conserved activity at 24 h [16–18% residual levels compared with 11% in cells transfected with the wild-type (Fig. 3)]. The most extensively phosphorothioated siRNA proved to be cytotoxic, resulting in ~70% cell death compared with mock-transfected cells (measured as the expression level of the control mRNA GAPDH). This siRNA species was therefore not included in the further analysis. The remaining siRNA species were evaluated for increased persistence of silencing by analysing TF mRNA expression 5 days after a single transfection of 100 nM siRNA. At this point, TF expression in cells transfected with wild-type siRNA had recovered almost completely (80% residual expression compared with mock-transfected cells) (data not shown). In cells transfected with the most extensively 2′-*O*-methylated siRNA (M2+4), however, strong silencing was still evident (32% residual expression). The less extensively modified siRNA species (P2+2, M2+2), although less effective than M2+4, consistently resulted in lower TF expression 5 days post-transfection (55–60%) than the wild-type. Fewer modifications (P1+1, P0+2, M1+1, M0+2, A0+2) did not improve the silencing effect 5 days post-transfection. Time-course experiments demonstrated that the wild-type siRNA was still the most effective 3 days post-transfection, when silencing was relatively unimpaired. However, silencing drops off at a

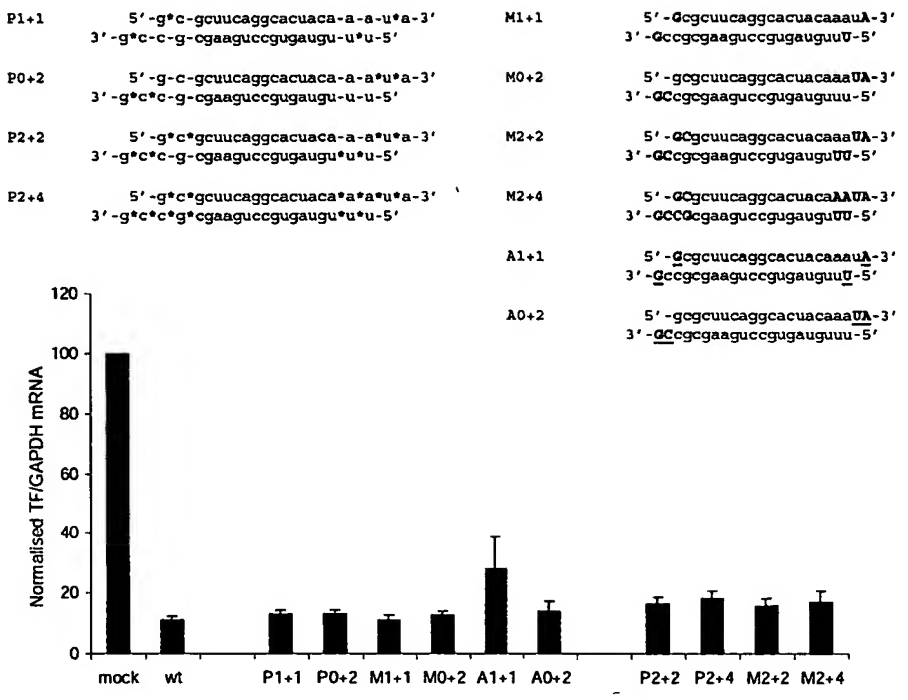

Figure 3. Activity of chemically modified versions of the siRNA hTF167i. Non-modified ribonucleotides are in lower case. Phosphorothioate linkages are indicated by asterisks (*), while 2′-*O*-methylated and 2′-*O*-allylated ribonucleotides are in normal and underlined bold upper case, respectively. Expression of TF and GAPDH mRNA was determined 24 h post-transfection of HaCaT cells. Experiments were performed and analysed as in Figure 2. Data are averages + s.d. of at least three independent experiments.

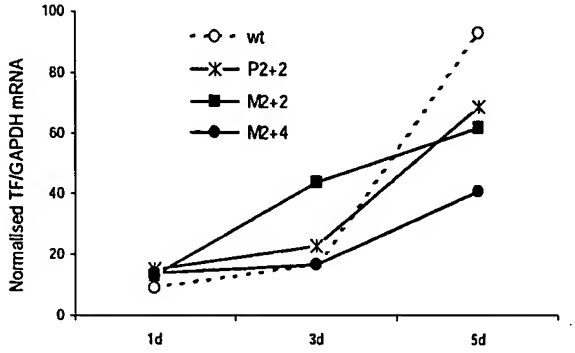

Figure 4. Persistence of TF silencing by chemically modified siRNAs in HaCaT cells harvested 1, 3 and 5 days after a single transfection of 100 nM siRNA. Medium was replaced every second day. Data are from one representative out of three independent experiments.

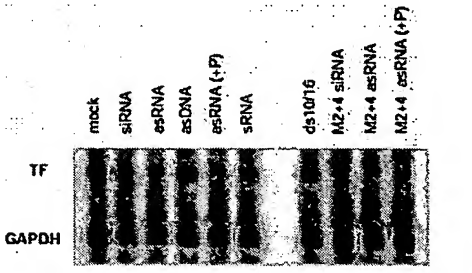

Figure 5. Levels of TF and control mRNA (GAPDH) in cells 24 h after transfection with various siRNA derivatives (see Figs 1 and 3). Cells were transfected with 100 nM siRNA duplex or 200 nM single-stranded RNA or DNA. RNA samples phosphorylated prior to transfection are indicated with '(+P)'. Phosphorylation was by polynucleotide kinase (New England Biolabs), followed by phenol/chloroform extraction and desalting on G25 Sephadex Quick-Spin columns (Roche).

much higher rate thereafter for the unmodified siRNA (Fig. 4), possibly due to a faster depletion of the intracellular siRNA pool.

Comparison of the effect of duplex and single-stranded antisense siRNA in whole cells

The antisense strand of siRNA is incorporated into RISC in HeLa cell extracts and supports RISC-specific target RNA cleavage, although at lower efficiency than the siRNA duplex (35,36). The antisense RNA was shown to silence transgene expression in a dose-dependent manner (36). We investigated whether antisense siRNA could silence endogenous gene expression with an efficiency comparable to duplex siRNA at moderate concentrations of the antisense oligo. HaCaT cells were transfected in parallel with 100 nM siRNA or 200 nM of the antisense strand, with or without pre-phosphorylation of the oligo (Fig. 5). In our quantitative assay the antisense RNA was as efficient as the siRNA duplex in depleting endogenous TF mRNA. A clear cleavage product was seen for both agents. The observation that antisense RNA exhibits almost full activity most likely reflects the high efficiency and excess capacity of this particular siRNA species. For the slightly less efficient chemically modified siRNA M2+4, on the other hand, there is a clear difference in depletion rate between the duplex

siRNA and antisense siRNA (Fig. 5), suggesting that higher concentrations are required for maximum effect of this antisense RNA. Phosphorylation prior to transfection did not improve the efficacy of either antisense strand, consistent with recent observations that single-stranded RNA can be phosphorylated *in vivo* (36).

DISCUSSION

This work has demonstrated tolerance of a highly effective siRNA, hTF167i, for a wide range of mutational and chemical modifications, as well as for removal of the sense strand from the duplex. Although allyl-modification of both terminal nucleotides resulted in some loss of activity, a total of six modifications in the form of either methylation of the sugar moiety or thiolation of the backbone were well tolerated, causing only a marginal reduction in the maximal silencing observed with the unmodified siRNA. Toxicity was, however, observed with longer stretches of phosphorothioates, but not with the same level of methyl-modification. SiRNA with 2+4 2′-*O*-methylated terminal nucleotides (M2+4) demonstrated both conserved initial activity and its increased duration in time-course experiments. These active end-methylated siRNAs are in possible contrast to the observations by Elbashir *et al.* (32) that fully 2′-*O*-methylated siRNAs are inactive. While full substitution of either strand with 2′-deoxynucleotides destroys siRNA activity (31–33), up to four deoxynucleotide modifications in the 3′ ends were found to support good activity in an *in vitro* cleavage assay (32). Taken together, the above data suggest the existence of different degrees of tolerance for chemical modification of siRNAs.

We find that single-stranded antisense siRNA can be highly effective in depleting endogenous gene expression at relatively moderate concentrations. Although antisense siRNA is still less effective than the corresponding siRNA duplex, this observation should help reduce the cost of selecting the best siRNA site, as the initial screening can be accomplished through the synthesis of the antisense strand only.

We have demonstrated a lower tolerance to mutations in the 3′ end of our most active siRNA. This bias cannot be due to differences in siRNA duplex stability as all mutations were G-C inversions and thus to a first approximation energetically equal. The stability of the duplex between a mutated siRNA antisense strand and the mRNA should be more severely affected by a central than a peripheral target mismatch. Consistent with this, we observe that 5′ end mutations have a negligible effect on activity compared with more centrally located mutations. The observed bias might be linked to the proposed existence of a 'ruler' region in the siRNA which is primarily used by the RISC complex to 'measure' the target mRNA for cleavage (32). It was demonstrated that the 5′ end of the antisense strand of siRNA sets the ruler for target RNA cleavage. This is likely to occur by a physical interaction of RISC with this region of the siRNA, which should therefore be more sensitive to mismatches with the target RNA. The universality of this observation is currently being investigated, using siRNAs targeting other sites in TF. The identification of a region of generally increased sensitivity to the effect of mismatches with the target mRNA, would be of great importance for the potential use of siRNAs to specifically target transcripts of disease-associated alleles in various dominant-negative disorders.

Highly diverging effects of mutations on siRNA activity have been reported. Jacque *et al.* (6) found that a single mismatch in siRNA targeting the HIV LTR resulted in partial loss of activity, while another siRNA targeting the HIV VIF exhibited almost full activity. Four mutations, however, abolished activity completely. Complete abolishment of activity has been reported by Gitlin *et al.* (8), Klahre *et al.* (37) and Garrus *et al.* (38), for siRNAs with 5, 6 and 7 mutations, respectively. Kisielow *et al.* (39) reported that a siRNA resulting in essentially complete knockdown of the expression of its target gene, was unable to inhibit the expression of a transgene with two non-contiguous mutations in the recognition sequence of the siRNA. The positions of these mutations correspond to our ds10/13 double mutant, which also exhibited low activity in our assay. A central double mutation, reported by Boutla *et al.* (31) and ourselves (33) to support partial activity, led to severe loss of activity for Yu *et al.* (40) and Wilda *et al.* (41), the latter using a siRNA with only 17 base pairs. On the other hand, abolishment of *in vivo* activity by a single mutation has been reported. Brummelkamp *et al.* (10), using a shRNA assumed to be processed to siRNA by Dicer (13), achieved inactivation by single mutations in either the second or ninth position from the putative 5′ end of the shRNA. Gitlin *et al.* (8), argued the case for single mutation inactivation more strongly by isolating siRNA-resistant poliovirus strains containing single mutations in the target site on the genomic RNA, corresponding to the sixth or ninth nucleotide of the siRNA, counted from the 5′ end of the sense strand. On balance, different siRNAs seem to be inactivated by mutations to different degrees, the outcome being at least in part target-sequence dependent.

In previous work (33) we established that the siRNA effect depends at least in some instances on the siRNA target position, mirroring earlier observations for ribozymes and antisense oligos (34,42,43). Further support for siRNA position effects has now emerged from several different sources. Several examples of inactive siRNAs in mammalian cells have recently been described (11,44,45), while a weak positional effect in *Drosophila* lysates can be inferred from published data (32). In *C.elegans*, Simmer *et al.* (46) managed to activate dozens of previously inactive dsRNA stretches, using the RNAi-sensitive rrf-3 negative strain, and in some cases created RNAi knockout phenotypes from genes that had hitherto not been responsive to dsRNA. Finally, Yang *et al.* (47) recently reported some inactive chemically synthesised and *in vitro* transcribed siRNA, as well as inactive shRNA. The authors demonstrated that the new technique of esiRNA, in which an overlapping set of siRNAs are produced *in vitro* by partial digestion with *Escherichia coli* RNase III, can be superior even to dsRNA. Processing of dsRNA by Dicer starts from a fixed end and proceeds in a sequential manner (3,48,49), producing a largely non-overlapping set of siRNAs. Cleavage of a dsRNA by *E.coli* RNase III will thus create a larger and more complete set of siRNAs than the non-overlapping set produced by Dicer from dsRNA. The higher activity of the esiRNA supports the argument that different siRNAs have different activities. Otherwise any set of siRNA from a dsRNA would have the same activity.

The inactivation of siRNA by mismatches has implications for the proposed function of RNAi as a defence system against retro-transposons and viruses. It is unclear why a viral defence mechanism should allow escape of a virus by a single mismatch. A differentiated population of siRNA with widely differing activities seems more likely. Some siRNAs with an intermediate activity can thus be more vulnerable to siRNA:target mismatch, while intrinsically stronger siRNAs have excess capacity and tolerate a single mismatch, as clearly exemplified by the tolerance exhibited to chemical and mutational modifications of hTF167i in the present study. This tolerance in highly active siRNAs should make viral escape more difficult, and our model is therefore consistent with both the published data and the proposed biological role of RNAi as a viral defence.

ACKNOWLEDGEMENTS

This work was supported by grants from the Norwegian Cancer Society, Health and Rehabilitation, and the Research Council of Norway (RCN) to H.P. T.H. is a fellow of RCN.

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EXHIBIT 4 of
EXHIBIT B

siRNA function in RNAi: A chemical modification analysis

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ABSTRACT

Various chemical modifications were created in short-interfering RNAs (siRNAs) to determine the biochemical properties required for RNA interference (RNAi). Remarkably, modifications at the 2′-position of pentose sugars in siRNAs showed the 2′-OHs were not required for RNAi, indicating that RNAi machinery does not require the 2′-OH for recognition of siRNAs and catalytic ribonuclease activity of RNA-induced silencing complexes (RISCs) does not involve the 2′-OH of guide antisense RNA. In addition, 2′ modifications predicted to stabilize siRNA increased the persistence of RNAi as compared with wild-type siRNAs. RNAi was also induced with chemical modifications that stabilized interactions between A–U base pairs, demonstrating that these types of modifications may enhance mRNA targeting efficiency in allele-specific RNAi. Modifications altering the structure of the A-form major groove of antisense siRNA–mRNA duplexes abolished RNAi, suggesting that the major groove of these duplexes was required for recognition by activated RISC*. Comparative analysis of the stability and RNAi activities of chemically modified single-stranded antisense RNA and duplex siRNA suggested that some catalytic mechanism(s) other than siRNA stability were linked to RNAi efficiency. Modified or mismatched ribonucleotides incorporated at internal positions in the 5′ or 3′ half of the siRNA duplex, as defined by the antisense strand, indicated that the integrity of the 5′ and not the 3′ half of the siRNA structure was important for RNAi, highlighting the asymmetric nature of siRNA recognition for initiation of unwinding. Collectively, this study defines the mechanisms of RNAi in human cells and provides new rules for designing effective and stable siRNAs for RNAi-mediated gene-silencing applications.

Keywords: RNAi; siRNA; human; nucleotide modification; GFP

INTRODUCTION

The evolutionarily conserved phenomenon RNA interference (RNAi), the process by which specific mRNAs are targeted for degradation by complementary short-interfering RNAs (siRNAs), has increasingly become a powerful tool for genetic analysis and is likely to become a potent therapeutic approach for gene silencing (for review, see Hammond et al. 2001; McManus and Sharp 2002). Consequently, understanding the mechanism of RNAi has become critical for developing the most effective RNAi methodologies for both laboratory and clinical applications. The general mechanism of RNAi involves the cleavage of double-stranded RNA (dsRNA) to short 21–23-nt siRNAs. This processing event is catalyzed by Dicer, a highly conserved, dsRNA-specific endonuclease that is a member of the RNase III family (Hammond et al. 2000; Zamore et al. 2000; Bernstein et al. 2001; Hamilton et al. 2002; Provost et al. 2002; Zhang et al. 2002). Processing by Dicer results in siRNA duplexes that have 5′-phosphate and 3′-hydroxyl termini, and subsequently, these siRNAs are recognized by the RNA-induced silencing complex (RISC; Hammond et al. 2000). Active RISC complexes (RISC*) promote the unwinding of the siRNA through an ATP-dependent process, and the unwound antisense strand guides RISC* to the complementary mRNA (Nykanen et al. 2001). The targeted mRNA is then cleaved by RISC* at a single site that is defined with regard to where the 5′-end of the antisense strand is bound to the mRNA target sequence (Hammond et al. 2000; Elbashir et al. 2001b). For RNAi-mediated mRNA cleavage and degradation to be successful, 5′-phosphorylation of the antisense strand must occur, and the double helix of the antisense-target mRNA duplex must be in the A form (Chiu and Rana 2002).

One highlighted difference between mammalian RNAi and RNAi in other eukaryotes is the lack of an amplification system for long-term persistence of RNAi in mammalian cells. For example, in *Drosophila*, ~35 molecules of dsRNA can silence ~1000 copies of the targeted mRNA per cell and can persist over the course of many generations (Kennerdell

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Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.5103703.

and Carthew 1998; Zamore 2001). In mammalian cells, RNAi only persists effectively for an average of ~66 h before the siRNA is likely diluted out over the course of several cell divisions (Chiu and Rana 2002). The amplification that is seen in flies and other lower eukaryotes can potentially be attributed to three factors. One is that the conversion of long trigger dsRNA to smaller 21–23-nt siRNAs by Dicer adds a degree of RNAi amplification, whereas in mammalian cells long trigger dsRNA invokes the interferon response that activates the protein kinase PKR (Stark et al. 1998). This suggests that only siRNA transfections successfully trigger RNAi in mammalian cells without other side effects, and thus, no amplification would take place through the processing of longer RNAs. A second factor in amplification is the presence of RNA-dependent RNA polymerase (RdRP), which has been found in plants, worms, fungi, and flies (Cogoni and Macino 1999; Dalmay et al. 2000; Sijen et al. 2001). RdRP has been postulated to amplify target mRNA, through a random, degradative PCR model (Lipardi et al. 2001; Nishikura 2001; Sijen et al. 2001), into dsRNA, which can be targeted by Dicer. However, no RdRP homologs have been found in mammalian cells, and the 3′-OH that is required for RdRP-dependent degradative PCR is not required for RNAi in mammalian cells (Chiu and Rana 2002; Schwarz et al. 2002; Stein et al. 2003), indicating that PCR-based amplification likely does not occur in mammals. A third factor in amplification may be the high enzymatic turnover rate of RISC* during the targeting and cleavage of mRNA (Hutvagner and Zamore 2002), which may add a degree of amplification to RNAi induction in all eukaryotes, including mammals. However, as the persistence of RNAi occurs for only a short period of time, finding methods for increasing the longevity of siRNAs in human cells will be fundamental for applying RNAi to laboratory and therapeutic applications.

To address this issue of siRNA stability for prolonging the duration of dsRNA-mediated gene silencing and to further dissect the mechanism of RNAi in human cells, various chemically modified nucleotides predicted to affect siRNA stability were incorporated into siRNAs to study whether specific modifications increased or decreased the efficacy and persistence of RNAi in vivo. The most important of these modifications was to the 2′-OH of the ribonucleotide that distinguishes RNA from DNA and is required for the nucleophilic attack occurring during the hydrolysis of the RNA backbone, the reaction catalyzed by degradative RNases. Our results showed that the 2′-OH was not required for RNAi, indicating that structural rather than chemical properties of siRNA–mRNA duplexes were the key to inducing RNAi and that RISC* did not require the 2′-OH for ribonuclease activity. 2′-modified siRNAs also increased the persistence of RNAi in human cells. Modifications that stabilized base-pairing interactions were also incorporated into the antisense strand of siRNAs and were able to initiate RNAi, signifying that this class of chemical modifications could be used to increase the targeting efficiency of siRNAs for mRNA target sequences and for allele-specific inhibition of gene expression.

Other chemical modifications affected the formation of the major groove of the A-form helix of the antisense-siRNA–target-mRNA duplex, and potentially disrupted H-bonds or sterically hindered protein contacts, most probably preventing the RISC* complex from stably interacting with the dsRNA duplex. These modifications completely abolished RNAi, demonstrating that an intact major groove in the A-form helix and stable RNA–protein interactions were required for RNAi in human cells. Finally, previous observations of psorelan cross-linked siRNAs implied that unwinding of siRNA occurred from the 5′-end of the antisense strand and that complete unwinding may not be necessary for effective RNAi (Chiu and Rana 2002). By using mismatched or chemically modified nucleotides on either the 3′ or 5′ half of the antisense strand within the siRNA duplex, we have shown here that RNAi depended on the integrity of the 5′, and not the 3′, half of the siRNA duplex, as defined by the antisense strand. Altogether, these results gave insight into the essential biochemical properties of functional siRNAs and how specific changes in the siRNA structure can affect the efficacy of RNAi. Furthermore, these studies present new methodologies for improving the stability and utility of siRNAs for future RNAi applications.

RESULTS

2′-OH is not required for siRNA to enter the RNAi pathway

Previous results showed that RNAi effects typically peaked between 42 and 54 h posttransfection, and targeted gene expression started to be restored by 66 h posttransfection (Chiu and Rana 2002). To determine if the duration of RNAi could be prolonged by increasing the half-life of siRNAs, various chemical modifications were made to nucleotides that affected siRNA stability. These modified siRNAs were then tested in an improved dual fluorescence reporter assay based on the original assay developed previously (Chiu and Rana 2002). Briefly, GFP and RFP were constitutively expressed from pEGFP-C1 and pDsRed2-N1, respectively. EGFP mRNA was targeted for RNAi using a 21-nt siRNA targeted to nucleotides 238–258 of the EGFP mRNA (Fig. 1A). The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of siRNA duplexes and normalized to that observed in the mock-treated cells. The sequence of EGFP siRNA and EGFP mRNA, the specific mRNA cleavage site, plus the structures of the chemically modified nucleotides are diagrammed in Figure 1. As outlined previously, the cleavage site was defined precisely 11 nt downstream of the target position complementary to the 3′-most nucleotide of

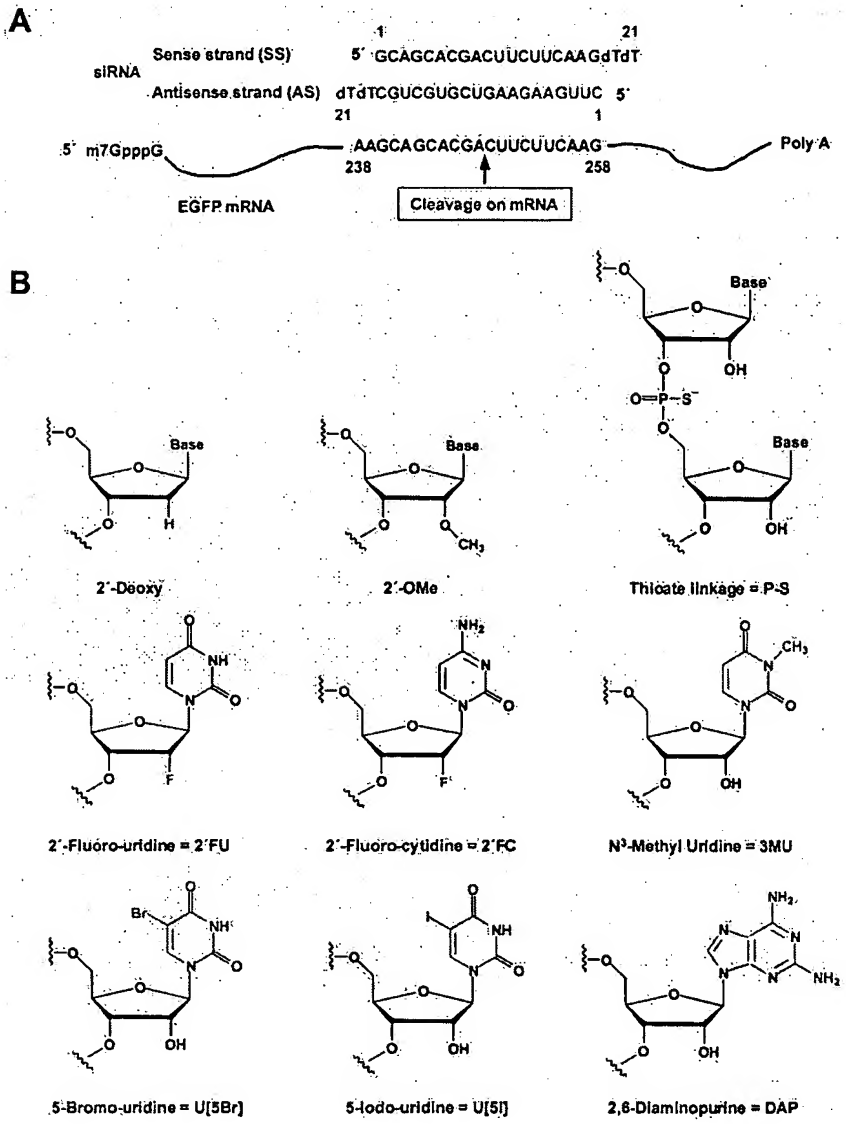

FIGURE 1. Structures of EGFP siRNA and chemical modifications. (*A*) Graphical representation of dsRNAs used for targeting EGFP mRNA. EGFP was encoded by the pEGFP-C1 reporter plasmid. siRNAs were synthesized with 2-nt deoxythymidine overhangs at the 3'-end. The position of the first nucleotide of the mRNA target site is indicated relative to the start codon of EGFP mRNA. The sequence of the antisense strand of siRNA is exactly complementary to the mRNA target site. (*B*) Structure and nomenclature of chemical modifications.

the antisense guide siRNA (Elbashir et al. 2001a). The specific chemical modifications, the particular siRNA strand(s) where modifications were made, and the effect of the chemically modified siRNA on RNAi activity are summarized in Table 1. The RNAi activity of siRNAs was evaluated with eight different siRNA concentrations (ranging from 1 to 200 nM). Each experiment was completed in duplicate and repeated twice.

The effects of modifying the 2'-OH of nucleotides on RNAi were studied by replacing uridine and cytidine in the antisense strand of siRNA with 2'-fluoro-uridine (2'-FU) and 2'-fluoro-cytidine (2'-FC), respectively, which have a fluoro group at the 2'-position in place of the 2'-OH (Fig. 1B). Where these modified 2'-FU, 2'-FC nucleotides reside in the siRNA sequence are highlighted in red in Figure 2A. Addition of a 2'-fluoro group should increase the stability of the siRNA by making the siRNAs less recognizable to RNases, thereby providing siRNAs protection from degradation (see below). When measured in the dual fluorescence assay, 2'-FU, 2'-FC siRNAs, modified only in the sense strand, only in the antisense strand, or in both strands, all showed decreased EGFP fluorescence when normalized to non-targeted RFP fluorescence that was comparable to the normalized decrease seen with wild-type siRNAs (Fig. 2; Table 1, rows 1–4). These results indicated that the 2'-OH was not required for RNAi and that nucleotides modified with 2'-fluoro groups could be used in siRNA constructs to successfully induce RNAi-mediated gene silencing.

To support the conclusion that the 2'-OH was not required for RNAi, adenine and guanine deoxynucleotides that inherently have 2'-H in place of the 2'-OH (Fig. 1B) were incorporated into the sense, antisense, or both strands of 2'-FU, 2'-FC-modified EGFP siRNAs to determine their effect on RNAi (Fig. 2A; green nucleotides). When 2'-FU, 2'-FC nucleotides were incorporated into the EGFP siRNA antisense strand with guanine and adenine deoxynucleotides at positions 9, 10, and 13, which base pair with nucleotides lining the cleavage site (Fig. 2A), EGFP RNAi effects were almost indistinguishable from wild-type levels (Fig. 2B; Table 1, row 5). This same antisense construct base-paired to 2'-FU, 2'-FC-modified sense strands also showed considerable EGFP silencing at ~64% (Table 1, row 6). In addition, siRNAs that had the entire antisense strand replaced with 2'-FU, 2'-FC, dATP, and dGTP nucleotides still showed moderate levels of RNAi activity at ~42%, or ~44% if the sense strand was also modified with 2'-FU, 2'-FC (Table 1, rows 7,8). All together, these results demonstrated that a 2'-OH group was not required for RNAi-mediated degradation and, even more specifically, was not required for nucleotides base-paired with nucleotides lining the mRNA cleavage site. There was, however, a limit on the extent to

TABLE 1. RNA interference mediated by chemically modified siRNAs

Row no.	EGFP siRNA	Sense strand	Antisense strand	RNAi activity (%)	RNAi activity (+ or −)
1	DS (WT)	Unmodified	Unmodified	93 ± 0.70	++++
2	SS/AS-2′-FU, FC	Unmodified	2′-FU, FC	83 ± 3.48	++++
3	SS-2′-FU, FC/AS	2′-FU, FC	Unmodified	92 ± 0.98	++++
4	DS-2′-FU, FC	2′-FU, FC	2′-FU, FC	83 ± 0.01	++++
5	SS/AS-2′-FU, FC + (9, 10, 13) dA, dG	Unmodified	2′-FU, FC + (9, 10, 13) dA, dG	85 ± 2.10	++++
6	SS-2′-FU, FC/AS-2′-FU, FC + (9, 10, 13) dA, dG	2′-FU, FC	2′-FU, FC + (9, 10, 13) dA, dG	64 ± 2.89	+++
7	SS/AS-2′-FU, FC + dA, dG	Unmodified	2′-FU, FC + dA, dG	42 ± 1.66	++
8	SS-2′-FU, FC/AS-2′-FU, FC + dA, dG	2′-FU, FC	2′-FU, FC + dA, dG	44 ± 0.60	++
9	SS/AS-Deoxy	Unmodified	Deoxy	0 ± 5.97	−
10	SS-Deoxy/AS	Deoxy	Unmodified	38 ± 2.95	+
11	DS-Deoxy	Deoxy	Deoxy	0 ± 0.01	−
12	SS/AS-2′-OMe	Unmodified	2′-OMe	16 ± 4.41	−
13	SS-2′-OMe/AS	2′-OMe	Unmodified	25 ± 1.75	+
14	DS-2′-OMe	2′-OMe	2′-OMe	0 ± 0.01	−
15	SS/AS-P-S	Unmodified	P-S	42 ± 6.03	++
16	SS-P-S/AS	P-S	Unmodified	62 ± 0.07	+++
17	DS-P-S	P-S	P-S	47 ± 0.03	++
18	SS/AS-2′-FU, FC + P-S	Unmodified	2′-FU, FC + P-S	22 ± 0.03	+
19	SS/AS-U[5Br]	Unmodified	U[5Br]	70 ± 1.88	+++
20	SS/AS-U[5I]	Unmodified	U[5I]	59 ± 11.2	+++
21	SS/AS-DAP	Unmodified	DAP	51 ± 0.57	++
22	SS-2′-FU, FC/AS-U[5Br]	2′-FU, FC	U[5Br]	31 ± 1.88	+
23	SS-2′-FU, FC, FC/AS-U[5I]	2′-FU, FC	U[5I]	42 ± 5.02	++
24	SS-2′-FU, FC/AS-DAP	2′-FU, FC	DAP	35 ± 7.69	+
25	SS/AS-3MU	Unmodified	3MU	0 ± 6.65	−
26	SS/AS-(11) 3MU	Unmodified	(11) 3MU	0 ± 1.71	−
27	SS/AS-(1, 2) mm	Unmodified	(1, 2) mm	35 ± 5.69	+
28	SS/AS-(18, 19) mm	Unmodified	(18, 19) mm	77 ± 2.00	+++
29	SS/AS-2′-FU, FC + (1-13) dA, dG	Unmodified	2′-FU, FC + (1-13) dA, dG	43 ± 0.09	++
30	SS-2′-FU, FC/AS-2′-FU, FC + (1-13) dA, dG	2′-FU, FC	2′-FU, FC + (1-13) dA, dG	45 ± 2.23	++
31	SS/AS-2′-FU, FC + (9-19) dA, dG	Unmodified	2′-FU, FC + (9-19) dA, dG	91 ± 0.36	++++
32	SS-2′-FU, FC/AS-2′-FU, FC + (9-19) dA, dG	2′-FU, FC	2′-FU, FC + (9-19) dA, dG	64 ± 0.42	+++

Summary of the specific chemical modifications analyzed, the particular siRNA strand(s) modified, and the effect of the chemically modified siRNA on RNAi activity in HeLa cells. RNAi activity was quantified by the dual fluorescence assay and is presented as the inhibition efficiency of target gene (EGFP) expression when cells were treated with 50 nM modified siRNAs. For comparison, the RNAi activity of unmodified, or wild-type, duplex siRNA (DS) normalized to 93% was designated (++++). Modified siRNAs assigned (++++) showed >80% RNAi activity, (+++) showed 60%–80%, (++) showed 40%–60%, and (+) showed 20%–40%. Modified siRNAs showing <20% RNAi activity were considered nonfunctional (−) in the RNAi pathway. Each experiment measuring RNAi activity of siRNAs was completed in duplicate and repeated twice.

which deoxynucleotides could substitute for ribonucleotides because replacing the entire siRNA sense strand with deoxynucleotides decreased EGFP gene silencing to ~38% inhibition, and replacing either the antisense strand or both strands entirely with deoxynucleotides completely abolished EGFP RNAi (Fig. 2B; Table 1, rows 9–11). Nonetheless, these results collectively showed that nucleotides with either 2′-F or 2′-H groups can selectively replace ribonucleotides within the siRNA sequence to effectively induce RNAi.

An interesting result was seen by modifying the 2′-OH to a bulky methyl group to create 2′-OMe nucleotides that were incorporated into sense, antisense, or both strands of EGFP siRNAs (Fig. 1B). This modification was hypothesized to improve RNAi efficacy because 2′-OMe groups are thought to increase RNA stability by inducing an altered RNA conformation that is more resistant to nucleases (Cummins et al. 1995). This modification is also thought to increase RNA affinity for RNA targets and improve hybridization kinetics (Majlessi et al. 1998). Despite these potential benefits, 2′-OMe nucleotides incorporated into either the sense or antisense strand greatly diminished EGFP gene silencing to ~25% or ~16%, respectively, whereas double-stranded 2′-OMe-modified siRNAs completely abolished RNAi (Table 1, rows 12–14). These results indicated that the methyl group, as a bulky group, may severely limit the interactions between siRNAs, target mRNAs, and the RNAi machinery required for successfully mediating RNAi. It is worth noting that because the bulkiness of the methyl group would likely be the cause of decreased RNAi activity rather than the actual lack of the 2′-OH specifically, these studies still supported the conclusion that the 2′-OH was not required for RNAi.

In a final analysis of modifications that may potentially increase siRNA stability without disrupting RNAi potency,

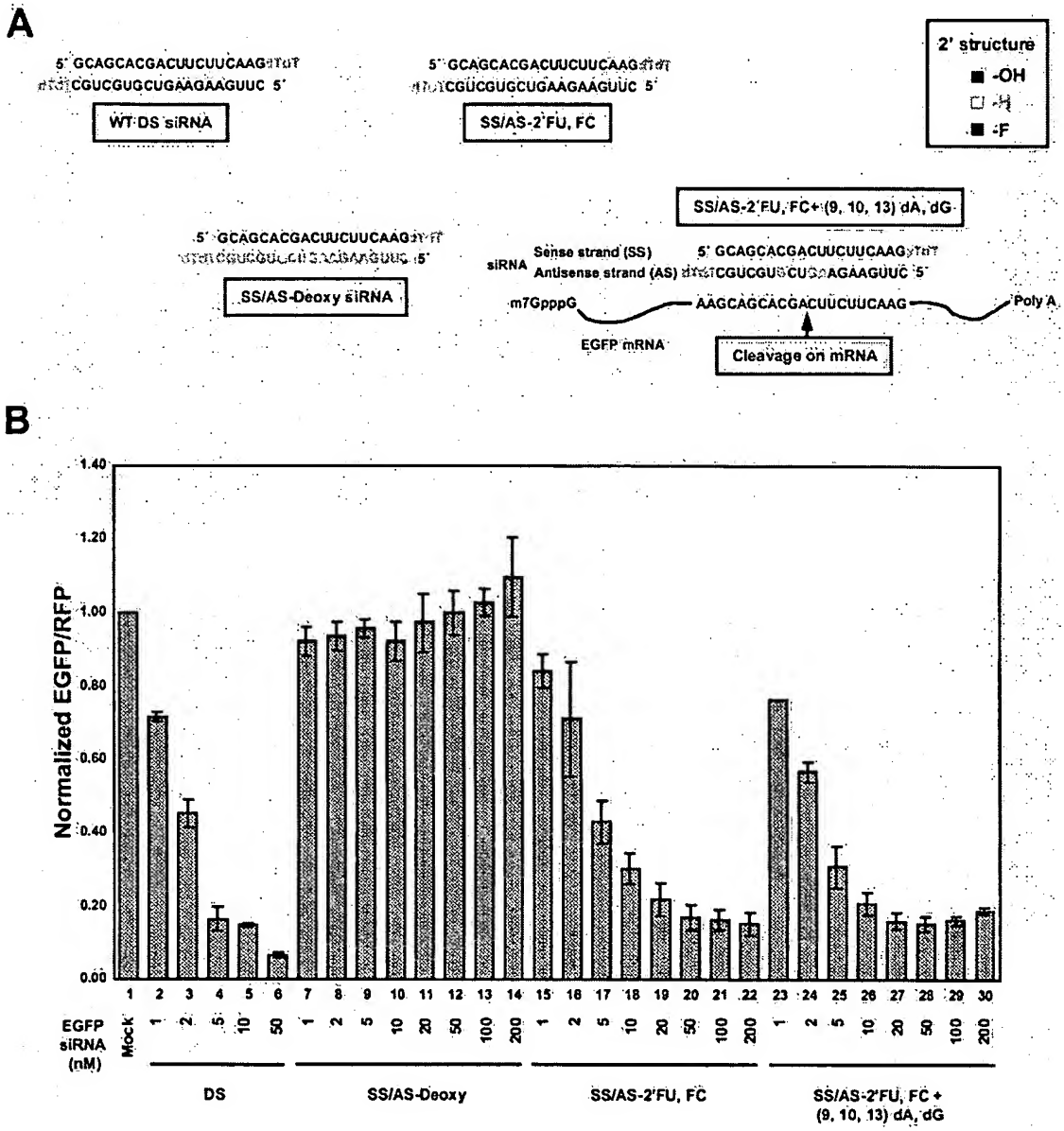

FIGURE 2. siRNA 2′-OH is not required to guide mRNA cleavage. (*A*) Sequence and structure of siRNA duplexes with modification at the 2′-position of the sugar unit. Nucleotides with 2′-hydroxyl groups (-OH) are black. Nucleotides with 2′-deoxy groups (-H) are cyan. Nucleotides with 2′-fluoro groups (-F) are red. The cleavage site on the target mRNA is also shown (red arrow). (*B*) Ratios of normalized GFP to RFP fluorescence intensity in lysates from modified siRNA-treated HeLa cells. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of EGFP siRNA duplexes with modifications at the 2′-position of the sugar unit. Normalized ratios at <1.0 indicate specific RNA interference effects. For comparison, results from unmodified duplex siRNA-treated cells are included.

a thioate linkage (P–S) was integrated into the backbone of the EGFP siRNA strand(s). P–S linkages were previously used in antisense methodology for increasing resistance to ribonucleases (for review, see Stein 1996) and, therefore, were postulated to enhance the stability of siRNAs. Incorporating the P–S linkages into the double-stranded siRNA sense strand led to moderate levels of RNAi activity (62% inhibition), whereas P–S linkages in either the antisense or both strands of the siRNAs led to just less than ~50% RNAi-induced inhibition (Table 1, rows 15–17). These results implied that the P–S modifications did not prohibit RNAi-mediated degradation and only moderately affected the efficiency of RNAi. Interestingly, incorporating 2′-FU, 2′-FC modifications into the antisense strand in addition to the added P–S linkages showed lower levels of EGFP gene silencing (Table 1, row 18), indicating that there was a synergistic effect that decreased but did not inhibit RNAi-mediated degradation when both the 2′-F groups and the

P–S linkages were incorporated into siRNAs.

Stability of modified siRNAs and the persistence of their RNAi activity in vitro and in vivo

As the above experiments showed that siRNAs modified with stabilizing 2′-FU, 2′-FC groups could effectively mediate RNAi to levels comparable to wild type, it was necessary to show that these modifications did in fact enhance siRNA stability. To measure the stability of siRNA in cell extracts, unmodified or 2′-FU, 2′-FC-modified EGFP antisense strand siRNAs 5′-labeled with [γ-^{32}P]ATP were annealed with sense strand siRNAs to form duplex siRNAs, which were then incubated in HeLa cell extracts. At various time points, siRNAs were extracted, analyzed on a 20% polyacrylamide gel containing 7 M urea, and visualized by phosphorimager analysis. Smaller siRNA degradation products were visualized in this analysis (data not shown), indicating that the lost of intact siRNA observed during these experiments was not caused by dephosphorylation of siRNAs. The top panel (a) of Figure 3A shows the stability of the various 2′-FU, 2′-FC-modified siRNAs as compared with wild-type siRNAs over time. Wild-type double-stranded siRNAs showed a steady loss of intact siRNAs over the course of the experiment, with only ~7% of the original concentration of intact siRNAs remaining after 1 h in extract (Fig. 3A[a], dark blue line). Intact modified or unmodified single-stranded antisense siRNAs were quickly lost over the time course and were virtually undetectable by 30 min in extract (Fig. 3A[a], black and red lines). This result showed that single-stranded modified siRNA was as susceptible to degradation as wild-type siRNA, indicating that single-stranded siRNAs, modified or unmodified, are inherently less stable than duplex siRNA. Double-stranded siRNAs with 2′-FU, 2′-FC modifications in either the antisense strand or both strands remained predominantly intact over the course of the experiment with ~68% or ~81%, respec-

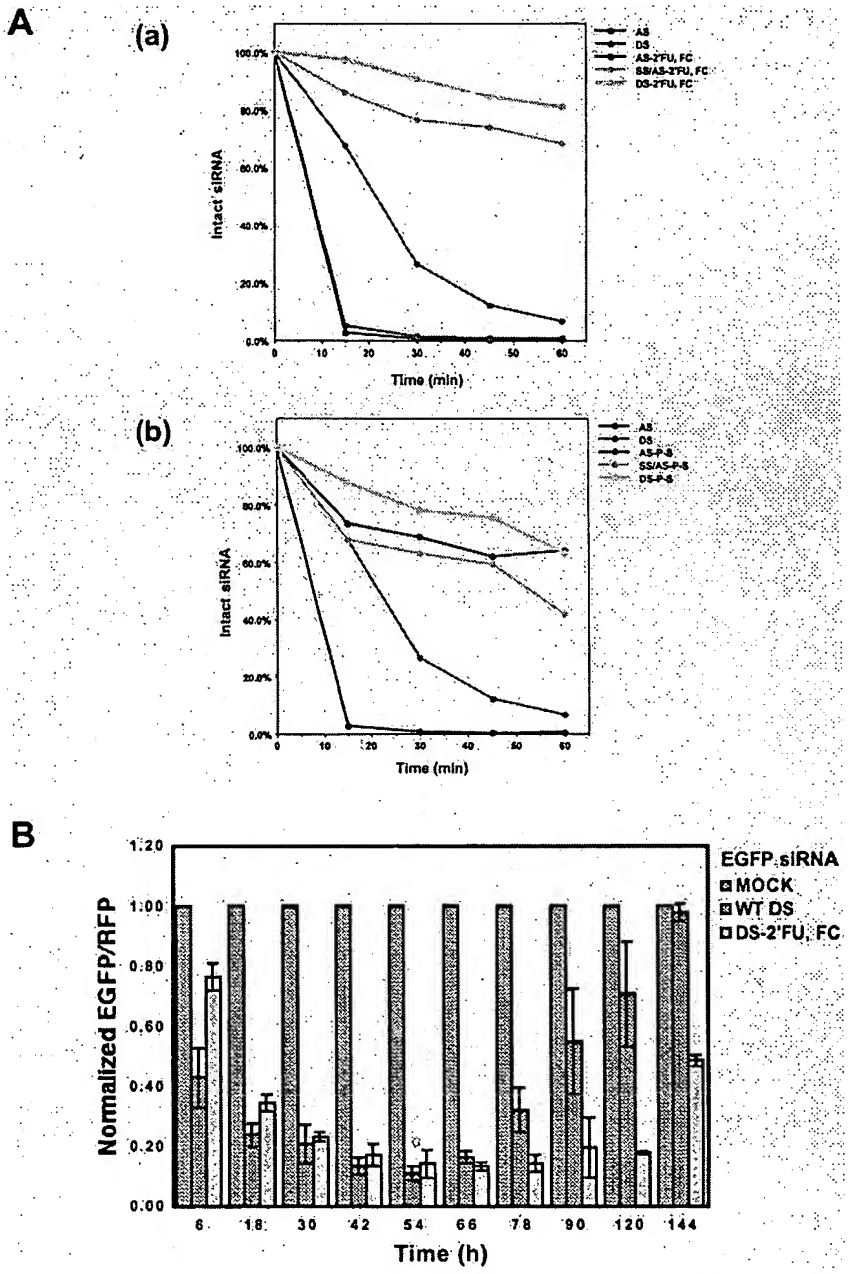

FIGURE 3. Extending the half-life of siRNA duplexes prolongs the persistence of RNA interference in vivo. (*A*) Comparing the stability of unmodified siRNAs with siRNAs containing 2′-fluoro-uridine and 2′-fluoro-cytidine (2′-FU, 2′-FC) modifications (*a*) and thioate linkage (P–S) modifications (*b*). Unmodified or modified EGFP antisense strand siRNAs (AS) were 5′-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinases. Duplex siRNAs were formed by annealing equal molar ratios of sense-strand (SS) siRNAs with the 5′-^{32}P-labeled antisense strand. To analyze siRNA stability in HeLa cell extract, 50 pmole of siRNA was incubated with 500 μg of HeLa cell extract in 50 μL of reaction mixture containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, and 10% glycerol. At various time points, siRNAs were extracted and analyzed on 20% polyacrylamide gels containing 7 M urea followed by phosphorimage analysis (Fugi). (*B*) Kinetics of RNAi effects of duplex siRNA with 2′-fluoro-uridine and 2′-fluoro-cytidine modification in HeLa cells over a 144-h time course. The fluorescence intensity ratio of target (GFP) to control (RFP) protein was determined in the presence of unmodified dsRNA (blue bars) and duplex siRNA with 2′-fluoro-uridine and -cytidine modifications (DS-2′-FU, 2′-FC, cyan bar) and normalized to the ratio observed in the presence of mock-treated cells (red bars). Normalized ratios at <1.0 indicated specific RNA interference.

tively, of the original siRNA population remaining intact throughout the duration of the experiment (Fig. 3A[a], green and light blue lines). These results indicated that the 2′-FU, 2′-FC modifications did, indeed, increase the stability of the siRNAs upon exposure to extract and that having these modifications in both strands provided the siRNAs with the most stability.

In a similar experiment, the stability of P–S-modified EGFP siRNAs was evaluated. Unmodified, double-stranded antisense siRNAs showed about the same rate of siRNA loss as described in the above experiment (Fig. 3A[b], dark blue lines). However, P–S-modified single-stranded antisense siRNAs demonstrated a markedly increased rate of stability over the course of the experiment, showing ~63% of the original siRNAs remaining intact after 1 h in extract as compared with 0% intact for single-stranded unmodified antisense siRNAs (Fig. 3A[b], black and red lines). The stability of double-stranded siRNAs with P–S modifications in both strands was comparable to the stability seen with the modified single-stranded antisense strand, with ~63% of the original siRNA population remaining intact after 1 h (Fig. 3A[b], light blue lines). Double-stranded siRNAs with P–S modifications in only the antisense strand showed weaker but still significant stability with ~42% of the original siRNA population remaining intact through 1 h in extract (Fig. 3A[b], green lines). These results showed that the P–S modifications increased the stability of the siRNAs and, most notably, increased the stability of both single- and double-stranded siRNAs.

These in vitro results indicated that siRNA stability is prolonged by these different modifications; however, it is important to note that these experiments address the general stability of siRNA in the context of endonucleases present in whole-cell extracts. Therefore, these experiments cannot distinguish whether the endonucleases affecting siRNAs in the in vitro assay would necessarily affect the stability of these various siRNAs in vivo. To address whether increased stability seen with modified siRNAs prolonged the duration of RNAi in vivo, RNAi, induced by unmodified and 2′-FU, 2′-FC-modified double-stranded EGFP siRNAs, was assayed in the dual fluorescence reporter assay over a period of 144 h. To visualize RNAi effects over an even longer period of time, HeLa cells were transfected with modified or unmodified siRNA and, 36 h later, transfected with dual fluorescence reporter plasmids; RNAi activity persisted but was tapering by 168 h (data not shown). Also, growth of cells containing modified siRNAs was comparable to cells containing wild-type siRNA, indicating that modified siRNAs were not affecting cell division (data not shown). Although 2′-FU, 2′-FC-modified EGFP siRNAs were slower to show RNAi effects by 6–18 h, maximal RNAi effects occurred by 42 h posttransfection for both modified and unmodified siRNAs. The maximal activity for both siRNAs was also in the same range, with both showing ~85%–90% inhibition of GFP expression. However, the RNAi effects observed over the period of 66–120 h revealed that the effect of modified siRNAs was much more persistent than that of unmodified siRNAs. By 120 h posttransfection, the effect of modified siRNAs still remained at ~80% inhibition of GFP expression but the effect of unmodified siRNAs had dropped to less than ~40% inhibition. Similarly, prolonged RNAi activity was observed with 2′-FU, 2′-FC-modified siRNAs targeting endogenous human Cyclin T1 mRNA when compared with wild-type siRNAs targeting Cyclin T1 (see Discussion; Y.L. Chiu and T.M. Rana, unpubl.). Altogether, these results strongly indicated that there was a direct link between the duration of the RNAi effects and siRNA stability in human cells. Furthermore, these results showed conclusively that siRNAs stabilized by chemical modifications, like the 2′-FU, 2′-FC modifications, can be used to effectively induce and significantly prolong RNAi-mediated gene silencing in vivo.

Modified siRNAs that stabilize A–U base-pair interactions can induce RNAi

In addition to incorporating modifications that affected the stability of siRNAs, nucleotides chemically modified to strengthen the base-pair interactions between two complementary bases were analyzed. In theory, increasing the stability of base-pair interactions may increase the targeting efficiency of siRNAs to target mRNA sequences. Increasing targeting efficiency may then induce more robust RNAi effects with siRNAs that are weaker at binding to their target sequence or have mismatched sequences, and thus, are not showing a high degree of RNAi. This type of approach may also be used to significantly inhibit expression of one allele over another when both alleles are present in the same cell. To bolster base-pairing interactions, 5-bromo-uridine (U[5Br]), 5-iodo-uridine (U[5I]), or 2,6-diaminopurine (DAP; Fig. 1B), which are modified nucleotides known to increase the association constant between A–U base pairs (Saenger 1984), were incorporated into siRNAs and tested in the dual fluorescence report assay. Double-stranded siRNAs having U[5Br], U[5I], or DAP modifications incorporated into the antisense strand were capable of inducing RNAi activity at levels of ~70% for U[5Br], ~59% for U[5I], and ~51% for DAP (Fig. 4; Table 1, rows 19–21). Interestingly, when 2′-FU, 2′-FC stabilizing modifications in the sense strand were combined with these modifications in the antisense strand, gene silencing was not as efficient as wild type in inducing RNAi. EGFP gene silencing was 31% for the 2′-FU, 2′-FC-modified sense siRNA base-paired with U[5Br]-modified, ~42% for U[5I]-modified, or ~35% for DAP-modified antisense siRNAs (Fig. 4; Table 1, rows 22–24). These results indicated that enhancing the interactions between base pairs through these siRNA modifications was a viable option for increasing mRNA targeting efficiency, but that there was a limit to how stable the base-pairing

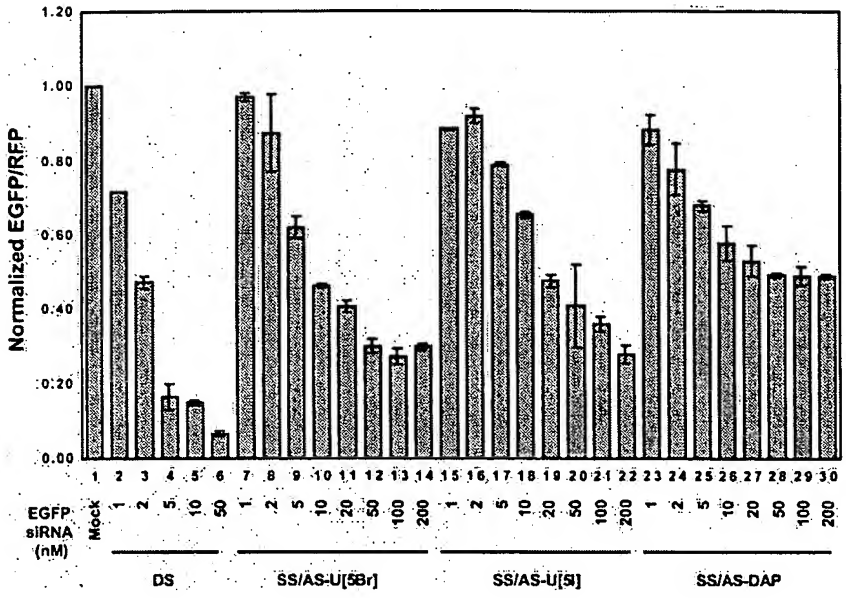

FIGURE 4. RNA interference mediated by siRNAs harboring modifications that stabilize base-pairing interactions. 5-Bromo-uridine (U[5Br]) or 5-iodo-uridine (U[5I]) replaced uridine or 2,6-diaminopurine (DAP) replaced adenine in siRNAs to stabilize base-pairing interactions. The activity of siRNAs with base modifications was quantified by the dual fluorescence assay. For comparison, results from unmodified duplex siRNA (DS, lanes 2–6)-treated cells are included.

interactions can be made before they interfere with siRNA unwinding (see Discussion).

The major groove of the A-form helix is required for RNAi

Previously, the A-form helix was shown to be required for the mechanism of RNAi, as 2-nt bulges that distort A-form helices between antisense siRNAs and target mRNAs abolished RNAi (Chiu and Rana 2002). To test whether the major groove of the A-form helix was required for RNAi, siRNAs were modified with N^3-methyl uridine (3MU) nucleotides that remove an H-bond donor at N^3-H. Structurally, the bulky N^3-methyl group would jut into the major groove of the A-form helix, potentially introducing a steric clash between base pairs. In addition, the presence of 3MU in the major groove may also introduce a steric clash between RNA and RNA-interacting proteins (Saenger 1984). Therefore, both steric hindrance and the loss of an H-bond donor by the addition of the N^3-methyl group should destabilize RNA–protein interactions in the major groove. 3MU-modified EGFP siRNAs introduced into HeLa cells completely abolished RNAi (Table 1, row 25). RNAi was also abolished if only one 3MU modification was introduced specifically at U11 of the antisense strand, which is one of the nucleotides that base pairs with A248 of the target EGFP mRNA cleavage site (Fig. 1A; Table 1, row 26). These results indicated that disrupting the functional groups of the major groove of the A-form helix formed by the antisense strand and its target mRNA specifically at the cleavage site inhibited RNAi. These data also indicated that the major groove was required for mediating RNAi and for RNA–RISC interactions that subsequently lead to mRNA cleavage.

Structural integrity of the 5′ half of siRNA duplex, as defined by the antisense strand, is important for mediating RNAi

Previous data using psoralen photochemistry suggested that complete unwinding of the siRNA duplex was not required for RNAi in vivo because psoralen cross-linked siRNAs did not completely abolish gene silencing (Chiu and Rana 2002). In describing these results, it was proposed that a single cross-linking event occurring near the 3′-end of the antisense strand still allowed for the initial unwinding of duplex siRNAs from the 5′-end, freeing enough of the nucleotides in the antisense strand to hybridize to the target mRNA and induce RNAi, even if unwinding was not complete. If this were the case, then unwinding of siRNAs must start from the 5′-end of the antisense strand, a conclusion supported by the fact that blocking either the 3′-end of the antisense siRNA strand or the 5′-end of the sense siRNA strand had no significant effect on RNAi activity (Chiu and Rana 2002).

If this 5′-to-3′ unwinding model was correct, sequences within the 3′ half of the siRNA duplex as defined by the antisense siRNA strand should be changeable without significantly interfering with RNAi. To test this hypothesis, EGFP siRNAs with mismatched base pairs at either the internal 5′ (nt 1, 2) or 3′ (nt 18, 19) positions within the duplex were introduced into the antisense strand (Fig. 5A, purple box, purple nucleotides). siRNAs with mismatches near the 5′ half of the duplex structure showed only ~35% inhibition in the dual fluorescence reporter assay, whereas mismatches at the 3′ half retained a significant level of gene silencing at ~77% (Fig. 5B; Table 1, rows 27–28). These results strongly indicated that the integrity at the 5′ half of the duplex, as defined by the antisense strand, was functionally more important than that at the 3′ half.

To explore this idea even further, 2′-FU, 2′-FC plus dATPs, dGTPs were incorporated within internal positions within the antisense-strand siRNAs predominantly at the 5′ (nt 1–13) or predominantly within the 3′ half (nt 9–19; Fig. 5A, red and cyan nucleotides). In the dual fluorescence

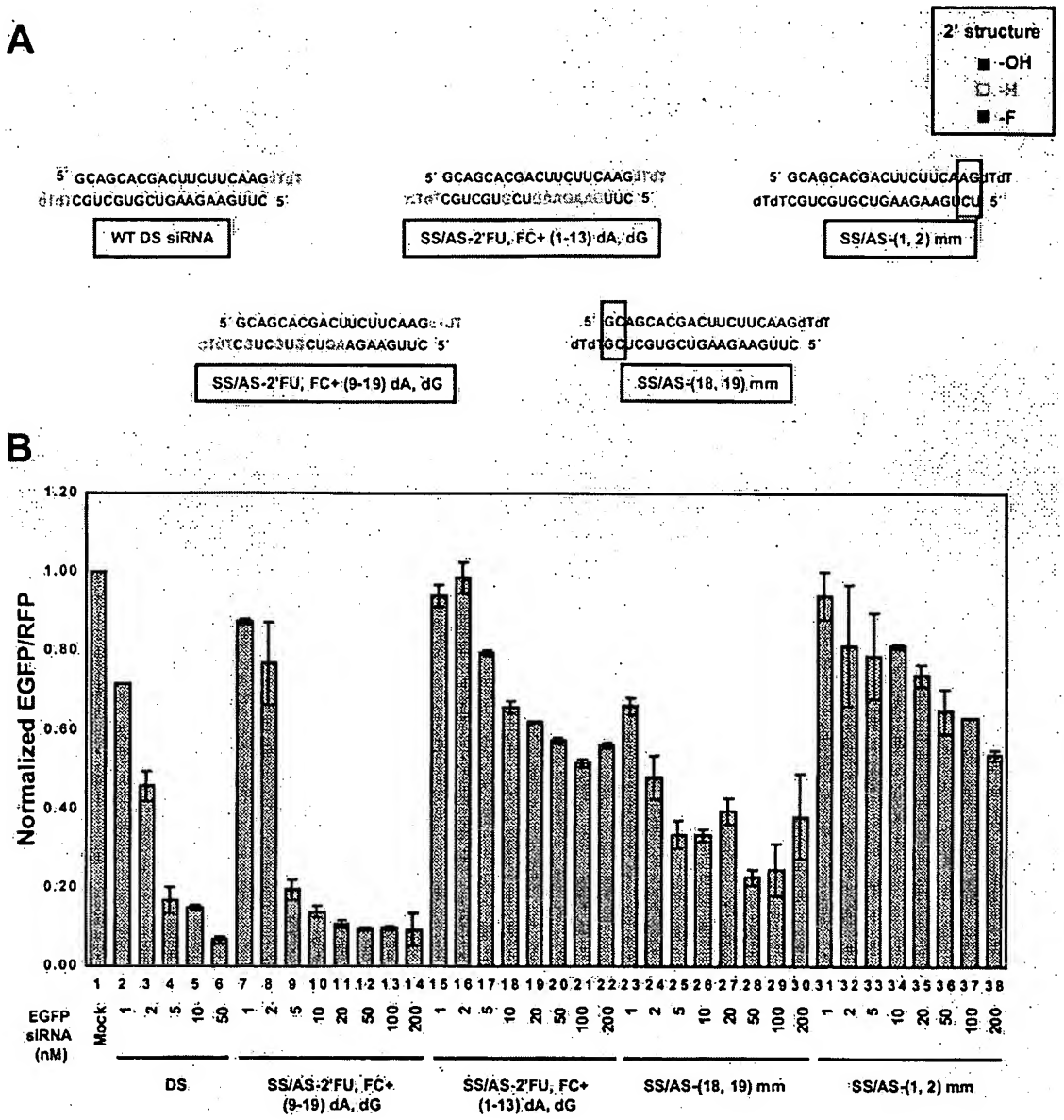

FIGURE 5. Structural integrity of the 5′ half of siRNA duplexes, as defined by the antisense strand, was functionally more important than at the 3′ half. (*A*) Graphical description of asymmetric requirement of duplex siRNA structure. The structure of unmodified (WT DS) siRNA duplexes, siRNAs with 2′-fluoro-uridine and 2′-fluoro-cytidine, 2′-deoxy modification at the 3′ half (SS/AS-2′-FU, 2′-FC + [9–19] dA, dG) or 5′ half (SS/AS-2′-FU, 2′-FC + [1–13] dA, dG) of the antisense strand, and siRNA duplexes with mismatches within the antisense 3′ half (SS/AS-[18,19] mm) or 5′ half (SS/AS-[1,2] mm) of the siRNA duplex are shown here. (*B*) Results from cells treated with duplex siRNAs with asymmetrically modified siRNA duplexes. For comparison, results from unmodified duplex siRNAs (DS, lanes *2–6*)-treated cells are included.

reporter assay, predominantly 5′-modified antisense (AS-2′-FU, 2′-FC + [1–13] dA, dG) EGFP siRNAs were only moderately effective, inducing RNAi at ~43%, or at 45% if the sense strand was also modified to 2′-FU, 2′-FC (Fig. 5B; Table 1, rows 29,30). However, predominantly 3′-modified and 5′-unmodified antisense (AS-2′-FU, 2′-FC + [9–19] dA, dG) siRNAs significantly induced RNAi activity at ~91%, or at 64% if the sense strand was also modified to 2′-FU, 2′-FC (Fig. 5B; Table 1, rows 31,32). These contrasting results indicated that sequence structure within the 5′ region of the antisense strand was more sensitive to modification than in the 3′region. All together, these data indicated that recognition of siRNA duplexes by an as-yet-unidentified RNA helicase occurs asymmetrically with the structure of the antisense 5′-end of the duplex preferentially distinguished from the 3′-end during the initiation of unwinding.

Modified siRNAs enter into the RNAi pathway in vitro

Although the dual fluorescence reporter assay did detect changes in EGFP gene expression with the modified siRNAs

created herein, it was possible that gene silencing was being induced by a mechanism other than RNAi-mediated degradative pathways. To test whether the targeted mRNA was, indeed, being cleaved upon exposure to modified siRNAs, an in vitro RNAi assay was performed to measure the cleavage of a ^{32}P-cap-labeled mRNA target upon incubation with modified siRNAs and HeLa cytoplasmic extract. Cleavage products were resolved on an 8% polyacrylamide-7 M urea gel. Mock-treated mRNAs did not show an observable cleavage product (Fig. 6, lane 1), but wild-type and all modified siRNAs that displayed gene silencing effects in vivo showed clearly visible cleavage products in vitro (Fig. 6, lanes 2,8–11,14–17). Furthermore, modified siRNAs that did not show any marked gene-silencing effects in vivo did not show any distinct cleavage products in the in vitro assay (Fig. 6, lanes 1,6,7,12,13), implying that the cleavage events observed were specifically dependent on functional siRNAs. These in vitro results provided a strong correlation between the in vivo gene silencing observed with the modified siRNAs and target mRNA degradation, indicating that the modified siRNAs were distinctly targeting mRNAs for cleavage and subsequent degradation through the in vivo RNAi pathway.

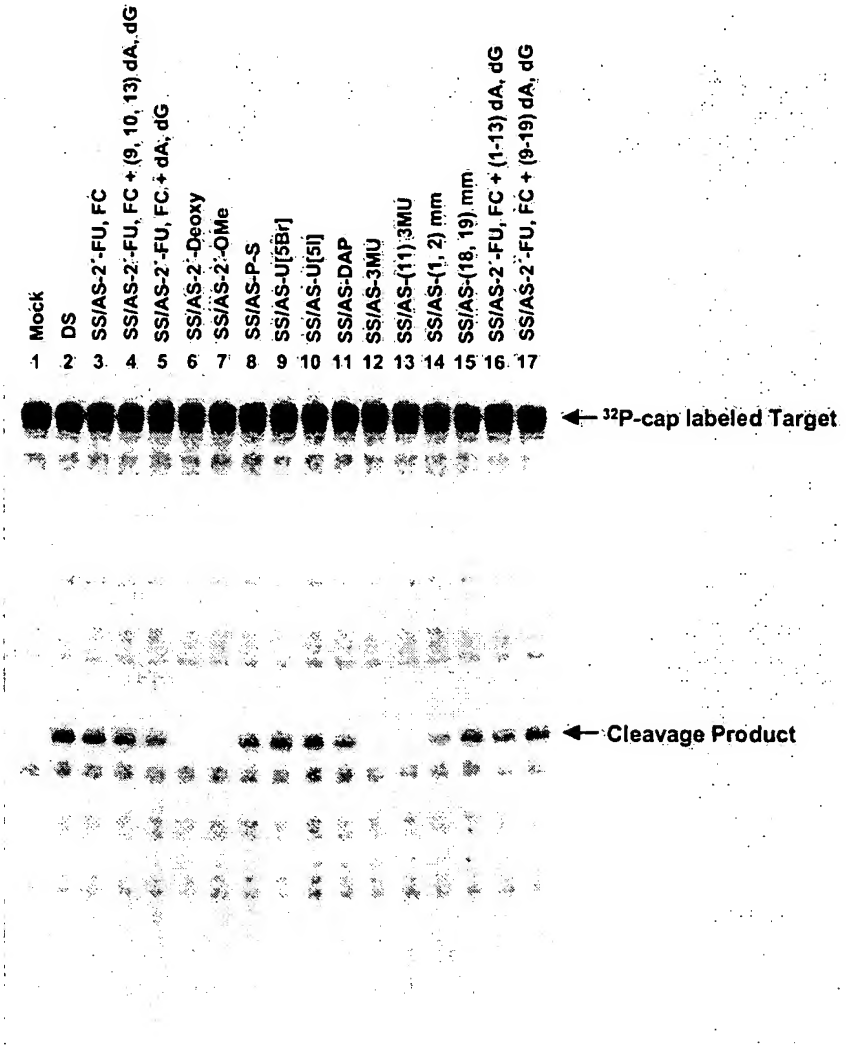

FIGURE 6. For analysis of siRNA-mediated target mRNA cleavage in vitro, 10 nM cap-labeled target EGFP mRNA was incubated with 100 nM siRNA and HeLa cytoplasmic extracts, as described in Materials and Methods. Reaction products were resolved on an 8% polyacrylamide-7 M urea gel. Arrows indicate the capped target EGFP mRNA and the 5′ cleavage product, which were expected to be 124 nt and 55 nt, respectively. The identity of the cleavage product was assigned according to RNase T1 partial digestion and a molecular weight marker of RNA (data not shown). The 3′ fragment is unlabeled, and therefore, invisible.

DISCUSSION

RNAi has moved toward the forefront of reverse genetic analysis in human cells for characterizing loss of gene function phenotypes and establishing connections between gene structure and function. In light of its versatility, understanding the detailed mechanism behind the RNAi phenomenon and developing methods to extend the limits of its current capabilities is crucial for implementing this methodology even further into the laboratory and therapeutic realms. By introducing various chemical modifications into siRNAs and measuring their effects on RNAi, this study revealed new insights into the mechanism of RNAi and outlined new approaches for increasing the efficacy of RNAi in vivo in human cells for use in future applications.

From these data, a more complete picture of the stepwise process of RNAi can be envisioned and is depicted in Figure 7. In the first step of RNAi induction, the 5′-ends of the siRNA duplex are phosphorylated, resulting in the formation of an siRNA–RISC complex. The data presented here showing the asymmetric nature of unwinding then indicate an ATP-dependent event during which siRNA is unwound from the 5′-end of the antisense strand and RISC is activated. Following RISC activation, the antisense strand of the unwound siRNA guides the siRNA–RISC* complex to the target mRNA. The guide antisense strand base pairs with the target mRNA, forming an A-form helix, and the RISC* protein complex recognizes the major groove of the A-form

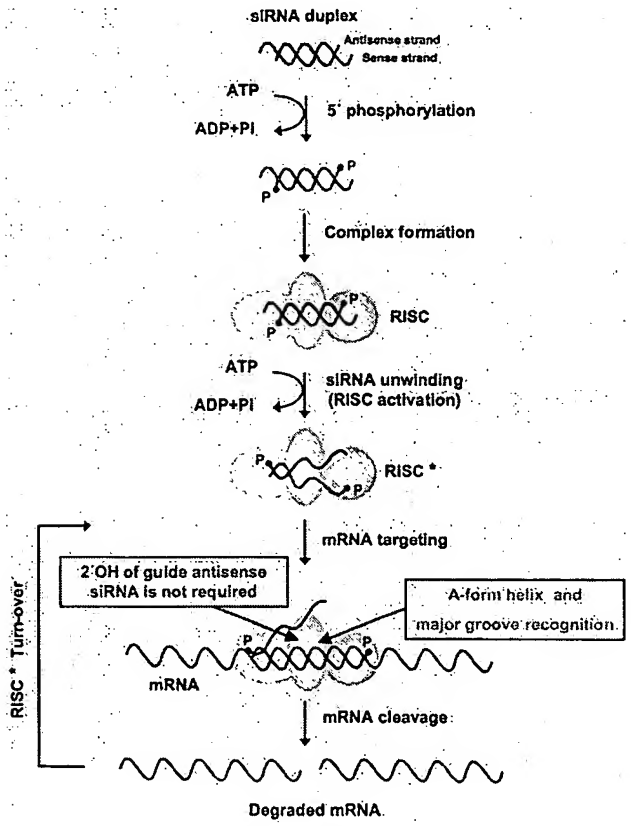

FIGURE 7. Model for RNAi in human cells highlighting the requirement of the A-form helix and major groove for mRNA cleavage with the steps not requiring the RNA 2′-OH of the guide antisense siRNA. See text for details.

helix, an event that occurs independently of the RNA 2′-OH of the guide antisense siRNA. In the final step of this process, the target mRNA is cleaved by RISC*, which is another event that occurs independently of the 2′-OH of the guide antisense siRNA. RISC* is then recycled to catalyze another cleavage event.

The requirement for the A-form helix supercedes the requirement for the 2′-OH in RNAi

Several important mechanistic findings were presented here that not only more clearly defined the mechanism of the RNAi pathway, but will also increase the utility of RNAi in various applications. Our results showing that the 2′-OH was not essential for RNAi have several important implications for the structural and catalytic elements required for the RNAi pathway. Remarkable functional implications were that the RNAi machinery does not require the 2′-OH for recognition of siRNAs, and the catalytic ribonuclease activity of RISC does not involve 2′-OH groups of the guide antisense RNA. Another consequence of this discovery was that a variety of chemical groups, including fluoro or deoxy groups, could substitute for the 2′-OH in siRNAs, indicating that no distinguishing chemical specificity was required for RNAi at the 2′-position. These findings would imply that other properties of the siRNA–mRNA duplexes, such as core structural elements, were essential for siRNA. If helical structure was the key to RNAi induction, then the A-form helix that forms between siRNAs and the target mRNA would, indeed, be required for RNAi, as was previously shown (Chiu and Rana 2002). Furthermore, the 2′-fluoro or combined 2′-fluoro, deoxy-modified antisense siRNAs lacking the 2′-OH would have to competently form an A-form helix to induce RNAi as shown here. This will likely turn out to be the case because 2′-fluoro-modified RNA–RNA hybrids were previously reported to exhibit an A-form helical conformation (Cummins et al. 1995; Luy and Marino 2001), lending significant merit to the idea that helical structure strongly influences RNAi efficiency. Still another implication of these particular results was that alternate chemical groups at the 2′-position that allow the A-form helix to be retained but help siRNAs evade recognition by RNases can increase siRNA stability and prolong RNAi effects induced in vivo.

It was previously shown in *Caenorhabditis elegans* and *Drosophila* extracts that completely substituting one or both siRNA strands with deoxynucleotides abolished RNAi (Parrish et al. 2000; Elbashir et al. 2001b), and those observations were consistent with the data presented here. The failure of true DNA–RNA hybrids to induce RNAi most plausibly relates to the argument that structure, and thus the A-form helix, was an essential determinant for RNAi induction. Based on circular dichroism spectra, DNA–RNA hybrids displayed characteristics that were intermediate between A- and B-form helices (Cummins et al. 1995). Following the contention that the A-form helix was an absolute requirement for RNAi induction, 2′-deoxy siRNA–mRNA target duplexes would not be recognized by the RNAi machinery because they would not be forming the proper A-form helical structure. Therefore, RNAi would not be induced by DNA–RNA hybrids, as has been observed. It is also worth mentioning that microRNAs (miRNAs) induce posttranscriptional gene silencing (PTGS) through the same pathway as RNAi but, ultimately, only inhibit translation machinery instead of inducing RNA degradation, the event that defines RNAi. The only observable difference between the two mechanisms is that RNAi requires the A-form helix, but miRNA-induced PTGS does not, as miRNAs often mismatch with their target mRNAs, forming a bulge that would distort the helical structure. This would indicate that the differences between the miRNA-induced silencing mechanism and siRNA-mediated RNAi may solely be attributable to differences in RNA–RNA helical structure, and further support a model in which helical structure was the sole determinant for whether RNAi was induced.

It was also previously reported that replacement of uridine with 2′-FU, corresponding to one-fourth of the bases of long dsRNAs, elicited RNAi effects in *C. elegans*, whereas

deoxycytodine incorporated into long dsRNAs diminished RNAi effects (Parrish et al. 2000). However, exactly where these modified nucleotides fell within the sequence structure of RNAi-inducing siRNAs and whether these modified nucleotides in the longer RNAs corresponded to the mRNA cleavage site or major groove after being processed to siRNAs was not clear. It has also been reported that siRNAs in which 3′ overhangs and two of the 3′-end ribonucleotides were replaced with deoxyribonucleotides retained RNAi activity upon exposure to *Drosophila* extracts (Elbashir et al. 2001b). Presumably, replacing two of the 3′-end base-paired nucleotides with deoxynucleotides would not disrupt the overall A-form structure of the siRNA–mRNA duplex required for RNAi and would thereby allow RNAi induction.

Neither analyses in *C. elegans* nor in *Drosophila* extracts ascertained whether there was a distinct requirement for the 2′-OH for cleavage-site recognition and the cleavage event itself during RNAi induction. The results presented here demonstrated that exclusively using 2′-FU, 2′-FC modifications in siRNAs and selectively substituting in deoxyribonucleotides for nucleotides base-paired with the nucleotides lining the mRNA cleavage site, or even replacing the entire sequence of siRNA with a combination of 2′-fluoro- and 2′-deoxynucleotides, elicited RNAi induction. Therefore, it has now been definitively established that recognition of the mRNA-target cleavage site and subsequent cleavage did not require the 2′-OH of the antisense siRNA to induce RNAi. As a final point, the inhibitory RNAi effects seen with the bulky 2′-OMe modification, which was also shown previously with *Drosophila* (Elbashir et al. 2001b), did demonstrate that there were steric constraints on the types of 2′ modifications that would be amenable for inducing RNAi. As 2′-OMe modifications probably did not disrupt the A-form helix of the siRNA–mRNA duplex (Cummins et al. 1995), the methyl group may be sterically interfering with protein–RNA interactions, thereby preventing RNAi. Nevertheless, steric constraints notwithstanding, this analysis conclusively showed that the nonessential nature of the 2′-position could very much be exploited for improving the efficacy of RNAi in a variety of applications.

Improving the efficacy of RNAi using chemical modifications

Modifications like the 2′-fluoro and P–S linkages both increased the half-life of siRNAs upon exposure to cytoplasmic extracts, and in vivo studies with 2′-FU, 2′-FC siRNAs showed that increasing the half-life of siRNAs did, in fact, prolong the effects of RNAi. In addition, we have observed similar prolonged RNAi activity with silencing lasting over 90 h using these same modifications in siRNAs targeting the endogenous target, human Cyclin T1 (Y.L. Chiu and T.M. Rana, unpubl.). These observations demonstrated that the siRNA modifications studied here can be used to effectively silence endogenous human genes over prolonged periods of time. Our results also indicated that short-lived RNAi effects usually observed in human cells were caused at least in part by the degradation of siRNAs. Our findings that the stabilizing siRNA modifications still allowed for a substantial level of RNAi induction showed that these modifications will be invaluable for studying the phenotypic effects of prolonged gene silencing in cell culture or in increasing the long-term in vivo effects of siRNAs in clinical applications. Interestingly, the P–S-modified, single-stranded antisense strand did not show increased RNAi effects in the dual fluorescence reporter assay used here (data not shown), despite showing significantly increased stability (Fig. 3A[a]). Similarly, single-stranded antisense siRNAs modified with 2′-OMe or 2′-FU, 2′-FC did not cause RNAi efficiently (data not shown). This strongly indicated that siRNA stability was not the main reason that single-stranded antisense RNAi was not as effective in inducing RNAi as dsRNA. Nonetheless, creating P–S modifications in the siRNA backbone showed that stabilizing the siRNA backbone did not inhibit RNAi and signified that using chemical modifications that stabilized phosphate linkages was a viable option for prolonging RNAi effects.

Another option for increasing the efficacy of RNAi was uncovered by the analysis of modifications that should enhance base-pairing interactions between antisense siRNA and targeted mRNA. DAP is a naturally occurring nucleobase that sometimes replaces adenine in phages like the cyanophage S-2L (Kirnos et al. 1977). Incorporation of DAP into RNA strands promotes the formation of three H-bonds between DAP and uridine, increasing the stability of interactions seen between A–U base pairs (Bailly and Waring 1998). U[5Br] and U[5I] have also been shown to have higher association constants when base-paired to A residues than to unmodified uridine (Saenger 1984). When any of these modifications were incorporated into siRNAs, RNAi was still quite efficient, indicating that modifications that stabilize base-pairing interactions can be used in designing siRNAs for various applications. It was also notable that siRNAs with 2′-fluoro modifications introduced into sense strands and base-paired with the DAP, U[Br], or U[5I] antisense strands had decreased RNAi efficiency. 2′-fluoro modifications have been shown to significantly increase the melting temperature between base pairs (Cummins et al. 1995). Consequently, the stabilizing effect on base-pairing interactions when both the 2′-fluoro and DAP, U[Br], or U[5I] modifications were present may have actually hindered the unwinding of the siRNA duplex. These results indicated that the lower rates of siRNA unwinding account for the observed decrease in RNAi activity.

Despite some minor limitations on how much the base-pair interactions can be stabilized, an application for using these types of modifications would be to increase the targeting efficiency of one mRNA sequence over another closely homologous but not identical sequence. Precedent

for this type of sequence discrimination was set by Haaima et al. (1997), who showed that DAP improved the ability of an oligomer to discriminate against mismatches. Translated to an RNAi application, these modifications may be useful for specifically targeting a mutant mRNA in a population of both mutant and wild-type mRNAs to recover a recessive wild-type phenotype. These modifications may also be useful in increasing binding affinity between target mRNAs and siRNAs that appear to have weak gene-silencing effects.

Other structural determinants for RNAi induction

Another structural facet of the RNAi mechanism was uncovered using the 3MU modification, which showed that the major groove of the A-form helix was required for RNAi. This finding builds on previous data showing that the A-form helix was required for RNAi (Chiu and Rana 2002). Together, these results indicated that the specific structure of the A-form helical RNA that forms the major groove and contains the mRNA cleavage site was important for recognition by the RNAi machinery. Conceivably, RNA–RISC* contacts depend on the structural integrity of the major groove for precise interactions and, ultimately, to initiate cleavage of the target. By disrupting the major groove, RISC* may no longer be able to interact or only weakly interacts with the siRNA–mRNA target duplex, thereby preventing mRNA cleavage. Alternatively, RISC* might still be able to interact with the destabilized RNA helix but not recognize the cleavage site within the major groove as the catalytic site if the conformation of the RNA helix and more specifically the major groove were altered.

The other structural property of siRNAs defined by these analyses was the asymmetric nature of siRNA unwinding. Initiation of siRNA unwinding from the 5′-end was previously indicated from the ability of single cross-linked siRNAs to still induce RNAi (Chiu and Rana 2002). By using mismatched or modified nucleotides on either the 3′ or 5′ half of the antisense strand within the siRNA duplex, it was shown here that RNAi depended on the integrity of the 5′, and not the 3′, half of the siRNA duplex, as defined by the antisense strand. Previous studies from our laboratory and from other groups have observed that mutations or modifications at the 3′-end overhang of the antisense strands are well-tolerated (Chiu and Rana 2002; Martinez et al. 2002; Amarzguioui et al. 2003; Holen et al. 2003). However, it is important to note that these studies did not address the role of nucleotides and the structure of siRNA at internal sequence positions. Here, we have specifically demonstrated the significance of nucleotides within the RNA duplex and their role in defining the structure of siRNA required for RNAi in vivo. These results indicated that, like RISC*, the RNA helicase, which has not yet been identified, also recognizes structural properties of the siRNA duplex as opposed to specific sequences of the RNA strands. This recognition appears to be asymmetric, with the structure of the antisense 5′ half of siRNA duplexes favored over the 3′ half, and is similar to how restriction enzymes can preferentially cleave the DNA backbone asymmetrically within a palindromic sequence. Further structural analysis of siRNAs to define what properties within the antisense 5′ half of the duplex contribute to the asymmetric nature of the duplex should help elucidate the specific structural elements required for duplex recognition by the RNA helicase for siRNA unwinding.

Our results showing that the modified siRNAs displayed effective RNAi in vivo and in vitro was also significant as it confirmed that the observed gene silencing was mediated by the RNAi pathway. These results also indicated that using chemical modifications that allow for efficient RNAi induction should work in the design of any given siRNA to increase its stability and capacity to specifically induce RNAi in vivo. Using these chemical modifications should take the field of RNAi quite a large step forward beyond the limits presently imposed by unmodified siRNAs with respect to long-term RNAi induction and targeting efficiency. One can imagine numerous applications for all of the chemical modifications used in this analysis, from studying prolonged RNAi effects on multiple genes in human cell cultures to opening up the door for long-term RNAi efficacy in therapeutic realms for curing a variety of genetic diseases.

From the data presented here, the mechanism of RNAi has been further elucidated and the groundwork for incorporating RNAi successfully into therapeutic realms has been laid out. We hope that future studies using the insight garnered here will not only help direct studies for further dissection of the mechanism of RNAi but will lead to new discoveries about gene function and facilitate the introduction of RNAi into vital clinical applications.

MATERIALS AND METHODS

siRNA preparation

The sequences of EGFP target-specific siRNA duplexes were designed as previously described (Chiu and Rana 2002). The 21-nt RNAs were chemically synthesized as 2′-bis(acetoxyethoxy)-methyl ether-protected oligonucleotides by Dharmacon. Synthetic oligonucleotides were deprotected, annealed, and purified; successful duplex formation was confirmed by 20% nondenaturing polyacrylamide gel electrophoresis (Chiu and Rana 2002).

Culture and transfection of cells

HeLa cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were regularly passaged at subconfluence and plated 16 h before transfection at 70% confluency. Lipofectamine (Invitrogen)-mediated transient cotransfections of reporter plasmids and siRNAs were performed in duplicate 6-well plates. A transfection mixture containing 0.16 μg of pEGFP-C1

and 0.33 μg of pDsRed2-N1 reporter plasmids (Clontech), various amounts of siRNA (1.0–200 nM), and 10 μL of lipofectamine in 1 mL of serum-reduced OPTI-MEM (Invitrogen) was added to each well. Cells were incubated in the transfection mixture for 6 h and further cultured in antibiotic-free DMEM. Cells were treated under the same conditions without siRNA for mock experiments. At various time intervals, the transfected cells were washed twice with phosphate-buffered saline (PBS, Invitrogen), flash-frozen in liquid nitrogen, and stored at −80°C for reporter gene assays.

Improved dual fluorescence assay

In an improved dual fluorescence reporter assay, EGFP-C1 encoded enhanced green fluorescence protein (GFP) and DsRed2-N1 encoded red fluorescence protein DsRed2 (RFP), a DsRed variant that has been engineered for faster maturation and lower unspecific aggregation. The extinction coefficient of DsRed2 is 43,800 M^{-1} cm^{-1}, and the quantum yield is 0.55, a significant quantitative increase when compared with the DsRed1 vector used in the dual fluorescence assay (Chiu and Rana 2002). To quantify RNAi effects, cell lysates were prepared from siRNA duplex-treated cells at 42 h posttransfection, as described previously (Chiu and Rana 2002). Then 240 μg of total cell lysate in 160 μL of reporter lysis buffer was measured by fluorescence spectrophotometry (Photo Technology International). The slit widths were set at 4 nm for both excitation and emission. All experiments were carried out at room temperature. GFP fluorescence in cell lysates was detected by exciting at 488 nm and recording from 498 to 650 nm. The spectrum peak at 507 nm represents the fluorescence intensity of GFP. RFP fluorescence in the same cell lysates was detected by exciting at 568 nm and recording from 588 to 650 nm. The spectrum peak at 583 nm represents the fluorescence intensity of RFP. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of siRNA duplexes and normalized to that observed in the mock-treated cells. Normalized ratios at <1.0 indicated specific interference.

Study of duplex siRNA stability in HeLa cell lysate

Unmodified or modified EGFP antisense strand siRNAs were 5′-labeled with [γ-^{32}P]ATP (3000 Ci/mM; ICN) by T4 polynucleotide kinases (New England Biolabs) at 37°C for 1 h and chase-kinased by adding 1 mM ATP at 37°C for 15 min. Free ATP and kinases were removed by the QIAGEN nucleotide removal kit. RNA was then purified by 20% polyacrylamide gel containing 7 M urea. Duplex siRNAs were formed by annealing equal molar ratios of unmodified or modified sense-strand siRNAs with the 5′-^{32}P-labeled antisense strand. Duplex formation was confirmed by 20% PAGE under native condition. We incubated 50 pmole of duplex siRNAs labeled at the 5′-end of the antisense strand with 500 μg of HeLa whole-cell extract in a 50-μL reaction mixture containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, and 10% glycerol. At various time points, 8-μL aliquots were mixed with 16 μL of loading buffer (0.01% bromophenol blue, 0.01% xylene cyanol, 98% formaldehyde, and 5 mM EDTA). The products were then denatured by heating at 95°C for 10 min and analyzed on 20% polyacrylamide gel containing 7 M urea followed by phosphorimage analysis (Fugi).

Preparation of HeLa cells cytoplasmic extract

Cytoplasm from HeLa cells was prepared following the Dignam protocol for isolation of HeLa cell nuclei (Dignam et al. 1983). The cytoplasmic fraction was dialyzed against cytoplasmic extract buffer (20 mM HEPES at pH 7.9, 100 mM KCl, 200 μM EDTA, 500 μM DTT, 500 μM PMSF, 2 mM $MgCl_2$, 10% glycerol). The extract can be stored frozen at −70°C after quick-freezing in liquid nitrogen. The protein concentration of HeLa cytoplasmic extract varied between 4 and 5 mg/mL as determined by a Biorad protein assay kit.

Preparation of cap-labeled target mRNA

For mapping of the target RNA cleavage, a 124-nt EGFP transcript, corresponding to nucleotides 195–297 relative to the start codon followed by the 21-nt complement of the SP6 promoter sequence, was amplified from template pEGFP-C1 by PCR using the 5′ primer GCCTAATACGACTCACTATAGGACCTACGGCGTGCA GTGC (T7 promoter underlined) and the 3′ primer TTGATTTAG GTGACACTATAGATGGTGCGCTCCTG-GACGT (SP6 promoter underlined). His-tagged mammalian capping enzyme was expressed in *Escherichia coli* from a plasmid generously provided by Stewart Shuman (Molecular Biology Program, Sloan-Kettering Institute, New York) and purified to homogeneity. Guanylyltransferase labeling was performed by incubating 1 nmole of transcripts with 50 pmole of His-tagged mammalian capping enzyme in the 100-μL capping reaction containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 2.5 mM $MgCl_2$, 1 U/μL RNasin RNase inhibitor (Promega), and [α-^{32}P]GTP at 37°C for 1 h. Reactions were chased for 30 min by supplementing GTP concentration to 100 μM. Cap-labeled target mRNAs were resolved on 10% polyacrylamide-7 M urea gel and purified.

In vitro target mRNA cleavage assay

siRNA-mediated cleavage of target mRNA in human cytoplasmic extract was performed as described (Martinez et al. 2002) with some modifications. siRNA duplexes were preincubated in HeLa cytoplasmic extract at 37°C for 15 min prior to addition of the 124-nt cap-labeled target mRNA generated as described above. After addition of all components, the final concentrations were 500 nM siRNA, 50 nM target mRNA, 1 mM ATP, 0.2 mM GTP, 1 U/μL RNasin, 30 μg/mL creatine kinase, 25 mM creatine phosphate, and 50% S100 extract. Incubation was continued for 1.5 h. Cleavage reactions were stopped by the addition of 8 volumes of proteinase K buffer (200 mM Tris-HCl at pH 7.5, 25 mM EDTA, 300 mM NaCl, and 2% [w/v] SDS). Proteinase K, dissolved in 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, and 50% glycerol, was added to a final concentration of 0.6 mg/mL. Reaction products were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), chloroform, and precipitated with 3 volumes of ethanol. Samples were separated on 8% polyacrylamide-7 M urea gels.

ACKNOWLEDGMENTS

We thank Tamara J. Richman for assistance in editing and critical evaluation of the work. We also thank B. Cullen, C. Mello, and P. Zamore for useful discussions and S. Shuman for kindly providing

reagents. This work was supported by grants from the NIH (AI41404, AI45466, and AI43198).

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Received May 28, 2003; accepted June 16, 2003.

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EXHIBIT 5 of

EXHIBIT B

D107

articles

Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs

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RNA interference (RNAi) holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called 'non-druggable' targets that are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies. The main obstacle to achieving *in vivo* gene silencing by RNAi technologies is delivery. Here we show that chemically modified short interfering RNAs (siRNAs) can silence an endogenous gene encoding apolipoprotein B (apoB) after intravenous injection in mice. Administration of chemically modified siRNAs resulted in silencing of the apoB messenger RNA in liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol. We also show that these siRNAs can silence human apoB in a transgenic mouse model. In our *in vivo* study, the mechanism of action for the siRNAs was proven to occur through RNAi-mediated mRNA degradation, and we determined that cleavage of the apoB mRNA occurred specifically at the predicted site. These findings demonstrate the therapeutic potential of siRNAs for the treatment of disease.

RNAi has been applied widely as a target validation tool in post-genomic research, and it represents a potential strategy for *in vivo* target validation and therapeutic product development[1]. *In vivo* gene silencing with RNAi has been reported using both viral vector delivery[2] and high-pressure, high-volume intravenous (i.v.) injection of synthetic siRNAs[3], but these approaches have limited if any clinical use. *In vivo* gene silencing has also been reported after local, direct administration (intravitreal, intranasal and intrathecal) of siRNAs to sequestered anatomical sites in models of choroidal neovascularization[4], lung ischaemia-reperfusion injury[5] and neuropathic pain[6], respectively. These reported approaches demonstrate the potential for delivery to organs such as the eye, lungs and central nervous system. However, there are no published reports of systemic activity for siRNAs towards endogenous targets after conventional and clinically acceptable routes of administration. A critical requirement for achieving systemic RNAi *in vivo* is the introduction of 'drug-like' properties, such as stability, cellular delivery and tissue bioavailability, into synthetic siRNAs.

Conferring drug-like properties on siRNAs

In exploring the potential of synthetic siRNAs to silence endogenous target genes, we found that chemically stabilized and cholesterol-conjugated siRNAs[7] have markedly improved pharmacological properties *in vitro* and *in vivo*. Chemically stabilized siRNAs with partial phosphorothioate backbone and 2'-*O*-methyl sugar modifications on the sense and antisense strands showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates. The conjugation of cholesterol to the 3' end of the sense strand of a siRNA molecule by means of a pyrrolidine linker (thereby generating chol-siRNA) did not result in a significant loss of gene-silencing activity in cell culture. Furthermore, unlike unconjugated siRNAs, a chol-siRNA directed to luciferase (chol-luc-siRNA) showed reduction in luciferase activity in HeLa cells transiently expressing luciferase, with a half-maximal inhibitory concentration (IC_{50}) of about 200 nM in the absence of transfection reagents or electroporation.

Binding of chol-siRNAs to human serum albumin (HSA) was determined by surface plasmon resonance measurement (data not shown). Unconjugated siRNAs demonstrated no measurable binding to HSA, whereas chol-siRNAs bound to HSA with an estimated dissociation constant (K_d) of 1 μM. Presumably because of enhanced binding to serum proteins, chol-siRNAs administered to rats by i.v. injection showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs. After i.v. injection in rats at 50 mg kg^{-1}, radioactively labelled chol-siRNAs had an elimination half life (two compartments), $t_{1/2}$ of 95 min and a corresponding plasma clearance (C_L) of 0.5 ml min^{-1}, whereas unconjugated siRNAs had a $t_{1/2}$ of 6 min and C_L of 17.6 ml min^{-1}. As measured by an RNase protection assay (RPA), chol-siRNAs showed broad tissue biodistribution 24 h after injection in mice. Although no detectable amounts of unconjugated siRNAs were observed in tissue samples, significant levels of chol-siRNAs were detected in liver, heart, kidney, adipose, and lung tissue samples. Together, these studies demonstrate that cholesterol conjugation significantly improves *in vivo* pharmacological properties of siRNAs.

Selection of apoB as an endogenous gene target

Apolipoprotein B is the essential protein for formation of low-density lipoproteins (LDL) in metabolism of dietary and endogenous cholesterol, and is the ligand for the LDL receptor[8]. Mouse apoB is a large protein of 4,515 amino acids and is expressed predominantly in liver and jejunum. apoB mRNA is subject to post-transcriptional editing, and the unedited and edited transcripts encode the full-length protein apoB-100, and a carboxy-terminal truncated isoform, apoB-48, respectively. In mice, editing of apoB mRNA occurs in both the liver and jejunum: apoB-48 is the predominant protein form in the jejunum and both apoB-48 and apoB-100 are expressed in the liver. Heterozygous knockout mice for apoB show a 20% decrease in cholesterol levels and are resistant to

diet-induced hypercholesterolaemia[9].

Serum levels of apoB, LDL and cholesterol correlate significantly with increased risk of coronary artery disease (CAD). A diminished number of functional LDL receptors on the cell surface, disrupting receptor-mediated removal of apoB-containing LDL from circulation, has been identified as the basis for familial hypercholesterolaemia (FH)[10]. Patients with homozygous and heterozygous FH have accelerated CAD leading to premature atherosclerosis and cardiac mortality. Conversely, patients with hypobetalipoproteinaemia have reduced levels of LDL and cholesterol and are at reduced risk for CAD[11]. Accordingly, lowering of serum cholesterol and LDL levels is a predominant clinical strategy for management of CAD and is achieved by modification of dietary sources of cholesterol and/or inhibition of endogenous cholesterol synthesis with pharmacological therapies. Notwithstanding significant improvements in the management of CAD with these approaches, millions of patients remain at significant risk for CAD and its clinical sequelae—acute coronary syndromes such as myocardial infarction and cardiac mortality—due to advanced atherosclerosis from intractably high levels of cholesterol and LDL. Clearly, new therapeutic strategies are needed. Accordingly, apoB, a protein not amenable to inhibition by conventional small-molecule- or protein-based therapeutics, was selected as a potential clinical target for development of siRNA therapeutics.

Using conventional bioinformatics, 84 siRNAs specific for both human and mouse apoB mRNA were designed and synthesized (data not shown). These apoB-siRNAs were screened for their ability to reduce apoB mRNA and protein levels, as measured by polymerase chain reaction with reverse transcription (RT–PCR) and enzyme-linked immunosorbent assay (ELISA), respectively, in HepG2 liver cells after transfection at a concentration of 100 nM. Five apoB-siRNAs were identified that reduced both mRNA and protein levels by >70%. Because exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum, two selected apoB-siRNAs (apoB-1-siRNA and apoB-2-siRNA) and one four-nucleotide mismatch control for apoB-1-siRNA (mismatch-siRNA) were stabilized at the 3′ end of the sense and antisense strands by phosphorothioate backbone modifications and additional incorporation of two 2′-*O*-methyl nucleotides at the 3′ end of the antisense strand. Chol-siRNAs were synthesized by linkage of cholesterol to the 3′ end of the sense strand via a pyrrolidine linker. Chol-apoB-1-siRNA was significantly more stable than unconjugated apoB-1-siRNA in human serum: gel electrophoresis showed >50% intact chol-apoB-1-siRNA after a 1 h incubation at 37 °C compared with <5% intact unconjugated apoB-1-siRNA. Similar data were obtained for chol-apoB-2-siRNA, although this siRNA was less stable than chol-apoB-1-siRNA. Dose response curves for the activity of conjugated and unconjugated apoB-specific and control siRNAs were measured in HepG2 cells using transfection. Two conjugated control siRNAs (chol-luc-siRNA and chol-mismatch-siRNA) showed no significant inhibition of apoB protein expression at concentrations as high as 30 nM. In contrast, three specific siRNAs (unconjugated apoB-1-siRNA, chol-apoB-1-siRNA and chol-apoB-2-siRNA) showed dose-dependent silencing of apoB protein expression based on apoB ELISA measurements—IC_{50} values of 0.5 nM, 5 nM and 8 nM were calculated, respectively.

In vivo studies with modified siRNAs

To demonstrate the ability of chol-apoB-siRNAs to silence apoB expression *in vivo*, experiments were first performed in C57BL/6

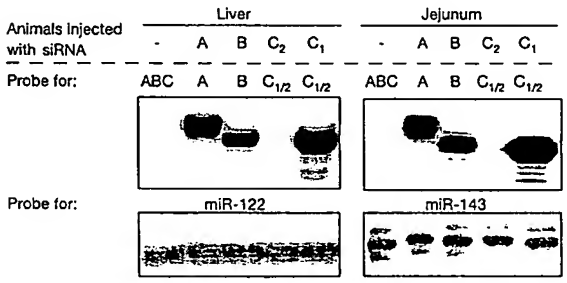

Figure 1 Biodistribution of siRNAs in liver and jejunum. An RPA was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals injected with saline (−), chol-luc-siRNA (A), chol-mismatch-siRNA (B), unconjugated apoB-1-siRNA (C_2) or chol-apoB-1-siRNA (C_1). Detection by RPA of endogenous miRNAs in liver (miR-122) and jejunum (miR-143) served as an internal loading control.

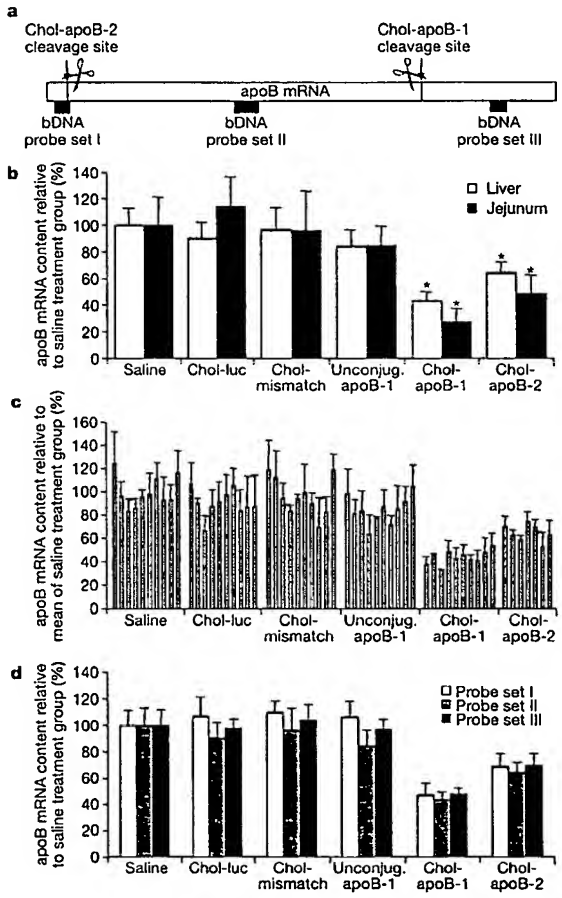

Figure 2 *In vivo* silencing of murine apoB mRNA by siRNAs in wild-type mice. Treatment groups comprised saline control ($n = 10$), chol-luc-siRNA control ($n = 10$), chol-mismatch-siRNA control ($n = 10$), unconjugated apoB-1-siRNA ($n = 10$), chol-apoB-1-siRNA ($n = 10$) and chol-apoB-2-siRNA ($n = 7$). bDNA measurements were performed with probe set II. Error bars represent the standard deviation (s.d.) of the mean. Statistical analysis was by analysis of variance (ANOVA) with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals. **a**, Schematic representation of the apoB mRNA illustrating the binding regions of three bDNA probe sets in relation to the two siRNA cleavage sites. **b**, Effects of siRNA administration on mean apoB mRNA levels. **c**, apoB mRNA levels from individual mice treated with saline or siRNAs. Data are mean values from three liver samples from each individual animal. **d**, Effects of siRNA administration on the reduction of apoB mRNA measured by bDNA assays using three different probe sets.

mice fed a normal chow diet. siRNAs were administered by tail-vein injection with normal volume (0.2 ml) and normal pressure. Biodistribution of siRNAs was assessed by RPA of siRNAs in tissue samples from liver and jejunum obtained 24 h after the last injection. Significant levels of chol-luc-siRNA, chol-apoB-1-siRNA and chol-mismatch-siRNA were detected in liver and jejunum (100–200 ng g^{-1} tissue for chol-apoB-1-siRNA), whereas levels of unconjugated apoB-1-siRNA were below our detection limit (Fig. 1). Levels of chol-apoB-2-siRNA were also detected but at levels approximately 10% of those observed for other chol-siRNAs.

The primary measure of RNAi-mediated effects is the reduction (that is, silencing) of the target mRNA. To measure silencing of apoB mRNA, we used a branched-DNA (bDNA) detection method and bDNA probes (Fig. 2a) to quantify apoB mRNA levels in liver and jejunum, two organs where apoB is known to be expressed. As shown in Fig. 2b, mice treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed statistically significant reductions (mean ± s.d.; 57 ± 6% and 36 ± 8%, respectively) in apoB mRNA levels in liver samples as compared with saline control ($P < 0.0001$). In jejunum tissue samples, mice injected with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed an even more substantial reduction in apoB mRNA levels of 73 ± 10% and 51 ± 13%, respectively, as compared with saline control ($P < 0.0001$). Individual animal results for apoB mRNA reduction in the liver are shown in Fig. 2c and demonstrate the consistent and robust effect observed for specific chol-siRNAs as compared with other treatment groups. Similar results were observed for apoB mRNA reduction in the jejunum from individual animals (data not shown). Owing to the extended length of the apoB mRNA, two additional probes at the distal ends of the apoB open reading frame (ORF) were designed. As measured with the three divergent probe sets, identical levels of apoB mRNA reduction were detected for animals treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA (Fig. 2d). These data suggest a uniform and rapid degradation of apoB mRNA after treatment with chol-apoB-siRNAs, and argue against the potential existence of truncated amino-terminal apoB protein fragments translated from incompletely degraded siRNA-cleavage products, as has been reported for ribozyme-mediated cleavage of apoB mRNA[12].

Silencing of the apoB mRNA would be expected to result in a corresponding reduction in apoB protein levels. An ELISA-based method specific for detection of apoB-100 protein was used to measure the effects of chol-apoB-siRNA treatment on plasma levels of apoB protein. In addition to the effects on apoB mRNA levels, treatment with chol-apoB-1-siRNA and chol-apoB-2-siRNA reduced plasma levels of apoB-100 protein 24 h after siRNA treatment by 68 ± 14% and 31 ± 18%, respectively, compared with levels in saline-treated control animals (Fig. 3). These results achieved statistical significance ($P < 0.0001$) for the group treated with the more potent and stable chol-apoB-1-siRNA. As the LF3 antibody used in this study recognizes only apoB-100, and not apoB-48, the observed apoB-100 reduction may underestimate the full effect of chol-apoB-1-siRNA at the protein level.

To confirm the physiological relevance of apoB mRNA silencing on lipoprotein metabolism, we characterized the effect of siRNA treatment and the resulting reduction of apoB protein levels on lipoprotein profiles and cholesterol levels. Using an NMR-based method, complete lipoprotein profiles were generated and concentrations of chylomicrons, very-low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) particles were calculated (Fig. 4a). As expected, HDL represented the predominant lipoprotein fraction in mouse plasma. Similar to results observed in heterozygous knockout mice for apoB[9], treatment with chol-apoB-1-siRNA resulted in a 25% reduction in HDL particle

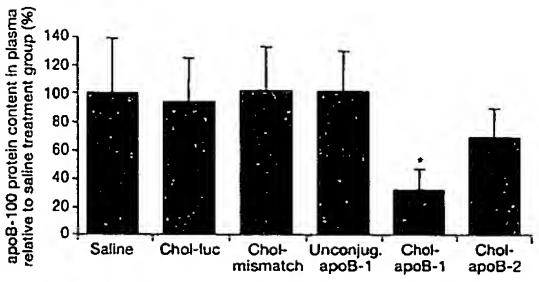

Figure 3 Effects of siRNA administration on apoB-100 protein levels. Average plasma levels of apoB-100 protein for the different treatment groups as measured by ELISA. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals.

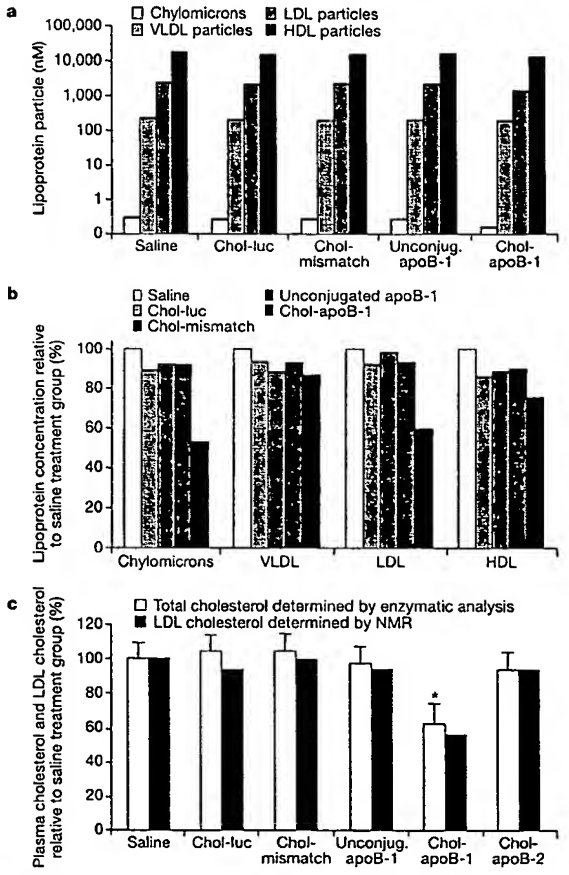

Figure 4 Therapeutic reduction of lipoprotein and cholesterol levels after siRNA treatment. a, Lipoprotein profile of pooled plasma samples from treatment groups determined by NMR analysis. b, Relative reduction of lipoprotein classes for the siRNA treatment groups normalized against the average levels of saline control group. c, Effects of siRNA administration on plasma cholesterol and LDL cholesterol. Plasma cholesterol was determined by enzymatic assay and LDL cholesterol calculated from NMR measurements. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. NMR data are based on single measurements of pooled plasma from treatment groups.

concentration (Fig. 4b). Furthermore, treatment of mice with chol-apoB-1 siRNA resulted in an almost 50% reduction of chylomicron levels and an approximately 40% reduction in LDL levels, whereas VLDL levels were not altered. Treatment with either of the control siRNAs did not change the lipoprotein profile significantly. In addition to reductions in lipoprotein concentrations, *in vivo* silencing of apoB by chol-apoB-1-siRNA led to a significant reduction ($37 \pm 11\%$; $P < 0.0001$) of total plasma cholesterol as compared with saline control animals (Fig. 4c). Treatment with the less potent chol-apoB-2-siRNA failed to show significant reductions in cholesterol, consistent with the reduced activity of this chol-siRNA on apoB mRNA and protein levels. Treatment with chol-apoB-1-siRNA also resulted in a 44% decrease in LDL-associated cholesterol, consistent with the effects observed on apoB protein levels. In aggregate, the effects on cholesterol reduction and lipoprotein profiles would be considered highly clinically significant in patients with hypercholesterolaemia, and actually exceed the level of cholesterol reduction observed in heterozygous apoB knockout mice[9].

To extend our findings of *in vivo* silencing by chol-apoB-siRNAs in normal mice, we performed an additional study in a human apoB transgenic mouse model[13]. These mice express human apoB-100 in liver and have elevated levels of apoB as compared with normal mice; when fed a high-fat diet, these mice develop severe atherosclerosis[14]. In our experiments, we administered saline, chol-mismatch-siRNA and chol-apoB-1-siRNA to apoB transgenic mice fed a normal chow diet. As shown in Fig. 5, chol-apoB-1-siRNA brought about a significant reduction of endogenous murine apoB expressed in both liver and jejunum tissue samples ($P < 0.0001$, relative to saline and chol-mismatch-siRNA treatment). Relative to the saline control, levels of murine apoB mRNA were reduced by $57 \pm 10\%$ in liver and $42 \pm 12\%$ in jejunum. In addition, chol-apoB-1-siRNA, which was selected in part owing to its sequence identity to both human and mouse apoB, showed significant silencing of the human transgene expressed in the liver, where human apoB mRNA was silenced by $60 \pm 10\%$ ($P < 0.0001$). In contrast to these effects, chol-mismatch-siRNA showed no effect on mouse or human apoB mRNA levels. These results confirm the effect of specific chol-siRNAs on apoB silencing in a different mouse model. Moreover, this specific chol-siRNA was shown to silence a transgenic human mRNA *in vivo*.

An important consideration for siRNA-mediated inhibition of gene expression is whether the observed effects are specific and not due to nonspecific "off target" effects[15] and potential interferon responses[16], which have been reported with siRNAs *in vitro* and other oligonucleotide-based approaches *in vivo*. In our experiments, the effects of apoB-specific, cholesterol-conjugated siRNAs were seen with two divergent siRNAs targeting separate sequence regions of the apoB mRNA. Furthermore, the *in vivo* silencing of apoB by these siRNAs was specific as neither an irrelevant siRNA (chol-luc-siRNA) nor a mismatch control siRNA (chol-mismatch-siRNA)—although present at comparable concentrations in liver and jejunum—mediated a significant reduction in apoB mRNA, plasma apoB protein levels, or total cholesterol. Finally, the silencing of apoB mRNA by chol-apoB-siRNAs in liver as measured by bDNA assay and normalization to GAPDH mRNA was also demonstrated with normalization to three other liver mRNAs, including factor VII, glucose-6-phosphatase and VEGF (Supplementary Fig. 1).

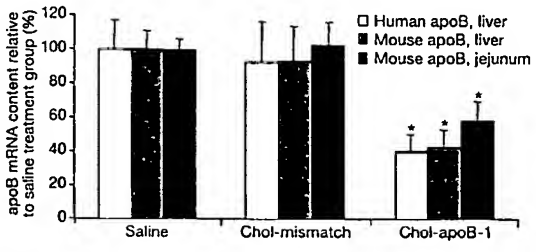

Figure 5 *In vivo* silencing of murine and human apoB mRNA in mice transgenic for human apoB. Reduction of human and mouse apoB mRNA levels in mice transgenic for human apoB that received saline ($n = 8$), chol-mismatch-siRNA ($n = 8$) and chol-apoB-1-siRNA ($n = 8$). Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. Error bars illustrate s.d. of the mean.

Determination of *in vivo* mechanism of action

To prove that the *in vivo* activity was due to siRNA-directed cleavage, we characterized specific mRNA cleavage products using a modified 5′-RACE (rapid amplification of cDNA ends) technique previously used to demonstrate microRNA (miRNA)-directed mRNA cleavage in plants[17] and mouse embryos[18]. As it relates to the specific cleavage of apoB mRNA by apoB-1-siRNAs, total RNA from mice in the different treatment groups was isolated, and then PCR was used to reveal fragments of the predicted length in animals receiving chol-apoB-1-siRNA treatment (Fig. 6a). Identity of the PCR products was confirmed by direct sequencing of the excised bands, which demonstrated that cleavage occurred at the predicted position for the siRNA duplex. Indeed, sequencing revealed cleavage after position 10,061 of the apoB ORF, exactly ten nucleotides downstream of the 5′ end of the siRNA antisense strand. Specific cleavage fragments were detected in both liver and jejunum of animals receiving chol-apoB-1-siRNA treatment (Fig. 6b). No fragments were detected in tissues of animals receiving control siRNAs (chol-luc-siRNA or chol-mismatch-siRNA) or saline. As expected, in this 5′-RACE experiment of apoB mRNA cleavage mediated by chol-apoB-1-siRNA, no fragments were detected in tissues from animals receiving the alternative apoB-specific siRNA (chol-apoB-2-siRNA). Notably, a low level of specific cleavage product was detected in the jejunum of animals receiving the unconjugated apoB-1-siRNA despite no evidence for significant knockdown of total apoB mRNA levels by this siRNA. This indicates

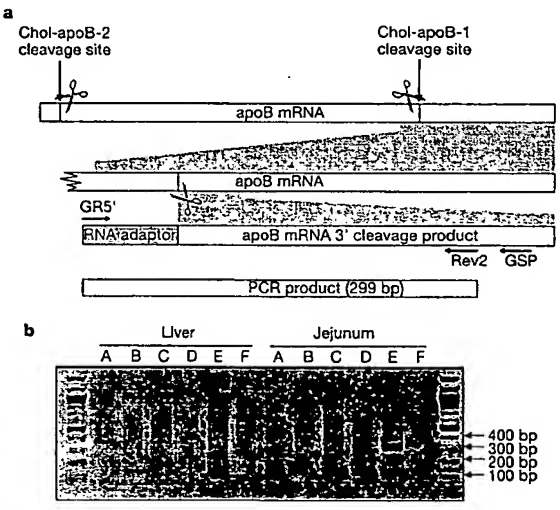

Figure 6 siRNA-mediated cleavage of apoB mRNA *in vivo*. a, Schematic representation of the apoB mRNA illustrating siRNA cleavage sites and RACE strategy to detect cleavage product. Cleaved mRNA ligated to an RNA adaptor was reverse transcribed using primer GSP. b, Agarose gel of 5′-RACE–PCR amplification, using the primer pair GR5′ and Rev2, showing specific cleavage products in liver and jejunum. Treatment groups are: A, saline; B, chol-luc-siRNA; C, chol-mismatch-siRNA; D, apoB-1-siRNA; E, chol-apoB-1-siRNA; F, chol-apoB-2-siRNA.

that some unconjugated apoB-1-siRNA is able to enter epithelial cells of the jejunum after systemic administration despite lacking cholesterol conjugation. Together, these data demonstrate that inhibition of apoB was achieved by an RNAi mechanism of action. To our knowledge, this is the first demonstration of silencing of an endogenous gene in mammals by a mechanism of RNAi-mediated degradation of the target mRNA.

Discussion

Our findings demonstrate that RNAi can be used to silence endogenous genes involved in the cause or pathway of human disease with a clinically acceptable formulation and route of administration by means of systemic delivery. In our study, we have shown that the mechanism of action for chemically modified siRNAs was by RNAi-mediated degradation of the target mRNA. Chol-apoB-siRNAs, but not unconjugated apoB-siRNAs, showed biological activity, demonstrating an important role for cholesterol conjugation of siRNAs to achieve systemic *in vivo* activity, and suggesting the opportunity to further optimize systemic activity through chemical conjugation strategies. Indeed, further optimization is warranted to achieve improved *in vivo* potency for chol-siRNAs at doses and dose regimens that are clinically acceptable. Nevertheless, these findings hold promise for the development of a new class of therapeutics that harnesses the RNAi mechanism. Of particular interest is the use of RNAi therapeutics to silence genes (such as the apoB gene) or mutated or variant alleles whose proteins are refractory to the discovery of traditional small molecules or biotherapeutic drugs. □

Methods

Synthesis of siRNAs

The siRNAs used in this study consisted of a 21-nucleotide sense strand and a 23-nucleotide antisense strand resulting in a two-nucleotide overhang at the 3′ end of the antisense strand. apoB-1-siRNA (ORF position 10049–10071): sense 5′-GUCAUCACAC UGAAUACCAA*U-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; chol-apoB-1-siRNA: sense 5′-GUCAUCACACUGAAUACCAAU*chol-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAc*a*C-3′; chol-mismatch-siRNA: sense 5′-GUGAUCAGACUCAAUACGAAU*chol-3′, antisense 5′-AUUCGUAUUGAGUCUGA UCAc*a*C-3′; chol-apoB-2-siRNA (ORF position 327–349): sense 5′-AGGUGUAUGGC UUCAACCCUG*chol-3′, antisense 5′-CAGGGUUGAAGCCAUACACCu*c*U-3′; chol-luc-siRNA: sense 5′-GAACUGUGUGUGAGAGGUCCU*chol-3′, antisense 5′-AGGAC CUCUCACACACAGUUc*g*C-3′. The lower-case letters represent 2′-*O*-methyl-modified nucleotides; asterisks represent phosphorothioate linkages.

RNA oligonucleotides were synthesized using commercially available 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers of uridine (U), 4-*N*-benzoylcytidine (C^{Bz}), 6-*N*-benzoyladenosine (A^{Bz}) and 2-*N*-isobutyrylguanosine (G^{iBu}) with 2′-*O*-*t*-butyldimethylsilyl protected phosphoramidites and the corresponding 2′-*O*-methyl phosphoramidites according to standard solid phase oligonucleotide synthesis protocols[19]. After cleavage and de-protection, RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography and characterized by ES mass spectrometry and capillary gel electrophoresis. RNA with phosphorothioate backbone at a given position was achieved by oxidation of phosphite with Beaucage reagent[20] during oligonucleotide synthesis. Chol-siRNAs were synthesized using the same protocols as above except that the RNA synthesis started from a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker (V.Ke., K.G.R. and M.M., unpublished data). For this support, the first nucleotide linkage was achieved using a phosphorothioate linkage to provide additional 3′-exonuclease stability. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed, and siRNAs were further characterized by native gel electrophoresis.

In vitro activity and stability assays

To determine *in vitro* activity of siRNAs, HepG2 cells were transfected with siRNAs using oligofectamine (Invitrogen) and siRNA concentrations ranging from 0.1, 0.3, 1, 3, 10 to 30 nM. apoB protein concentration was determined from cell culture supernatant by a sandwich ELISA capturing apoB with a polyclonal goat anti-human apoB antibody (Chemicon International). apoB detection was performed with a horseradish peroxidase-conjugated goat anti-human apoB-100 polyclonal antibody (Academy Bio-Medical Company). The remaining apoB protein content was calculated as the ratio of apoB protein concentration in the supernatant of cells treated with the apoB-specific siRNA duplex to the apoB concentration in the supernatant of cells treated with an unspecific control siRNA duplex. Mouse serum (Sigma-Aldrich Chemie GmbH) was used for stability assays. Double-stranded RNAs (5 μM) were incubated in 95% serum, and the mixture was incubated at 37 °C for various lengths of time (for example, 0, 15 or 30 min, or 1, 2, 4, 8, 16 or 24 h). siRNAs were isolated by hot phenol extraction in the presence of sodium dodecyl sulphate followed by ethanol precipitation. Re-suspended RNA samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide for 2 h at 45 mA. RNA bands were visualized by staining with the 'Stains-All' reagent (Sigma-Aldrich Chemie GmbH) according to the manufacturer's instructions.

In vivo silencing experiments

C57Bl/6 mice received, on three consecutive days, tail vein injections of saline or different siRNAs. All siRNAs were administered at doses of 50 mg kg^{-1} in approximately 0.2 ml per injection. Measurements of apoB mRNA, apoB protein levels, lipoprotein concentrations and plasma cholesterol content were performed 24 h after the last i.v. injection. Experiments were carried out in a blinded fashion. The same experimental design was used for experiments with the human apoB transgenic mice (1004-T hemizygotes, Taconic).

In vivo bioanalytical methods

An RPA, using radiolabelled probes complementary to the antisense strands, was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals treated with saline or siRNAs. RPA for endogenous miRNAs was used as a loading control for jejunum (miR-143, sequence 5′-UGAGAUGAAGCACUGUAGCUCA-3′) and liver (miR-122, 5′-UGGAGUGUGACAAUGGUGUUUG-3′).

The QuantiGene assay (Genospectra) was used to quantify the reduction of mouse apoB mRNA in liver and jejunum tissue after siRNA treatment. Small uniform tissue samples where collected 24 h after the last injection. Lysates from three tissue samples per animal were directly used for apoB and GAPDH mRNA quantification, and the ratio of apoB and GAPDH mRNA was calculated and expressed as a group average relative to the saline control group. Specific probes for detection of apoB mRNA levels were designed to the following regions of the apoB mRNA ORF: probe set I 83–385; probe set II 5,045–5,673; probe set III 12,004–12,411. Furthermore, apoB mRNA reduction in liver was quantified from purified (RNeasy mRNA isolation kit, Qiagen), pooled mRNA for each treatment group. As well as GAPDH, factor VII, glucose-6-phosphatase and VEGF mRNAs were also used for normalization.

ELISA was used to quantify the reduction of apoB-100 protein levels in mouse plasma after siRNA treatment. apoB-100 from plasma samples of individual animals was detected using the primary antibody LF3 against mouse apoB-100 (gift of S. Young; see ref. [21]). Levels were normalized to plasma volume and expressed as group averages relative to the saline control group.

Total cholesterol levels in the plasma were measured using the Cholesterol detection kit (Diasys). For NMR determination of the plasma lipoprotein profile a Bruker DRX 600 with cryoprobe head was used (LipoFIT Analytic GmbH). Single measurements of 500 μl mouse plasma (pooled from ten animals per treatment group) were performed. The lipoprotein subclass distribution was calculated from the NMR data by using computer algorithms that are based on human blood standards[22]. The particle number for lipoprotein classes was calculated based on the correlation of known particle size and composition with the experimentally determined NMR signal intensity. On the basis of this correlation, the cholesterol content in the LDL fraction was computed. The cholesterol values calculated from NMR data were confirmed by the presence of comparable levels of total cholesterol in plasma and HDL-cholesterol as determined by enzymatic assays.

5′-RACE analysis

Total RNA (5 μg) from pooled liver and jejunum samples from animals treated with different siRNAs was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer (GSP: 5′-CTCCTG TTGCAGTAGAGTGCAGCT-3′). To detect cleavage products, PCR was performed using primers complementary to the RNA adaptor (GR5′: 5′-CTCTAGAGCGACTGGAGCACG AGGACACTA-3′) and apoB mRNA (Rev2: 5′-ACGCGTCGACGTGGGAGCATGGAGGT TGGCAGTTGTTC-3′). Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of specific PCR products was confirmed by sequencing of the excised bands.

Received 2 September; accepted 20 October 2004; doi:10.1038/nature03121.

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Supplementary Information accompanies the paper on www.nature.com/nature.

Acknowledgements We thank P. Sharp for his advice and creative input. We are grateful to J. Maraganore and T. Ulich for their support and encouragement. We would like to thank S. Young for the LF3 anti-mouse apoB antibody; D. Bartel and S. Yekta for advice on the 5'-RACE assay; S. Young and M. Stoffel for valuable discussions; and LipoFIT Analytic GmbH and the Institute for Biophysics and Physical Biochemistry of the University of Regensburg for the characterization of lipoprotein particles by NMR. For technical assistance we thank P. Deuerling, F. Hertel, S. Leuschner, N. Linke, A. Müller, G. Ott, H. Schübel, S. Shanmugam, M. Duckman and C. Auger.

Competing interests statement The authors declare competing financial interests: details accompany the paper on Nature's website (http://www.nature.com/nature).

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